

UTILIZING ELECTROMYOGRAPHY TO IDENTIFY CAUSES OF EXHAUSTION IN PIGS
FED RACTOPAMINE-HCL

by

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Abstract

Pigs fed ractopamine-HCl (**RAC**) are more prone to fatigue and exhaustion when improperly handled. Wireless electromyography (**EMG**) can be used to directly measure median power frequency (**MdPF**) and root mean square (**RMS**) as indicators of action potential conduction velocity and muscle fiber recruitment, respectively. The objectives of this study were to determine the effect of RAC on exhaustion, EMG measures, and muscle fiber type characteristics when barrows were subjected to increased levels of activity. Thirty-four barrows were assigned to one of two treatments: a commercial finishing diet containing 0 mg/kg (**CON**) RAC or a diet formulated to meet the requirements of finishing barrows fed 10 mg/kg RAC (**RAC+**) for 35 d. After 32 d of feeding, barrows were walked around a circular track at 0.79 m/s until subjective exhaustion was reached. Time, distance, and speed were measured. Wireless surface EMG sensors were affixed to the *Deltoideus* (**DT**), *Triceps brachii lateral head* (**TLH**), *Tensor fasciae latae* (**TFL**), and *Semitendinosus* (**ST**) muscles. After harvest, samples of each muscle were collected for fiber type, succinate dehydrogenase, and capillary density analysis. Speed was not different ($P = 0.82$) between treatments, but RAC+ barrows reached subjective exhaustion quicker and covered less distance than CON barrows ($P < 0.01$). The end-point MdPF was not affected by the RAC diet. The RAC diet did not change end-point RMS values in the DT or TLH; however, the RAC+ barrows tended to have decreased ST and increased TFL end-point RMS values ($P < 0.07$). The percentage of type I fibers tended to be greater ($P = 0.07$) in RAC+ barrows, but the RAC diet tended to increase ($P = 0.07$) size of type I fibers and increase ($P = 0.03$) the size of type IIA fibers. Succinate dehydrogenase was not different between treatments. The RAC+ barrows had more ($P = 0.03$) capillaries per fiber than CON barrows. A diet containing RAC contributes to increased onset of subjective exhaustion, possibly due to rapid

loss of active muscle fibers and chronic loss of oxidative muscle fibers with no change in muscle metabolism.

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I can't wait to see what the next chapter of my life holds, but I feel confident that this experience has prepared me for whatever comes my way.

Dedication

To my husband Zach, my parents Kere and Ronda, and my cat Reagal. Without each of you, I would not be where I am today

Chapter 1 - Introduction

“Fatigued pig syndrome” is a loosely defined term used to describe swine fatigue during transport. This syndrome encompasses common welfare and economic concerns for swine producers and meat packers due to non-ambulatory pigs arriving at the packing plant after transport. Transport loss is described as any animal that dies or becomes non-ambulatory anytime during transport (Ritter et al., 2009) due to injury or fatigue (Fitzgerald et al., 2009). Non-ambulatory animals include both injured and fatigued pigs. A fatigued pig shows no sign of disease, injury, or trauma; however, they refuse to walk (Ritter et al., 2005) and often display open-mouth breathing, skin discoloration, muscle tremors, and abnormal vocalizations (Anderson et al., 2002). Bertol et al. (2002) and Ritter et al. (2006) suggest that fatigued pigs arriving at the plant may recover and walk on their own if allowed to rest for 2 to 3 h before harvest; however, Hamilton et al. (2004) reported a strong correlation ($r = 0.81$) between non-ambulatory and dead pigs. Although injured non-ambulatory pigs are classified in this group, fatigued pigs are also included. Therefore, it can be hypothesized that fatigued pigs might die before having the chance to rest after transport.

In an extensive review of transport losses in market weight pigs, Ritter et al. (2009) found that transport losses cost the swine industry approximately \$46 million annually. The authors explained how economic losses in the pork industry stem from multiple factors including dead pigs, non-ambulatory pigs, and carcass bruising. Finally, the authors categorized economic losses as either direct or indirect. The direct loss encompassed the price paid for the non-ambulatory or dead pigs minus the cost of the farrow-to-finish operation phase, which amount to \$105.03/pig and \$17.18/pig for dead and non-ambulatory pigs, respectively. Indirect losses were calculated as lost profit opportunities, which amounted to a loss of \$20.47/pig.

Fatigued pig syndrome can result from multiple factors. These factors include, but are not limited to, genetics, nutrition, health, weight, handling, environment, transport, and muscling (Anderson et al., 2002). Recently, ractopamine-HCl (**RAC**) has been studied to determine its effect on swine fatigue (James et al., 2013; Peterson et al., 2015; Puls et al., 2015). Ractopamine is a β -adrenergic agonist that is fed during the finishing phase of swine production to improve ADG, feed efficiency, and lean muscle growth (Anderson et al., 1987; Watkins et al., 1990; Moloney et al., 1991). Although this feed additive improves growth and carcass characteristics, it can also cause fatigue when pigs are handled roughly (James et al., 2013; Puls et al., 2015). Many scientists have used indirect measures, such as blood metabolites (James et al., 2013; Puls et al., 2015, Peterson et al., 2015) or subjective measures of visual observations (Marchant-Forde et al., 2003; Rocha et al., 2003; Puls et al., 2015), to determine fatigue when pigs were supplemented with RAC. While these studies have provided valuable insight into RAC stimulated fatigued pig syndrome, they have failed to directly identify the physiological processes within the muscle responsible for failure.

In human kinesiology research, electromyography (**EMG**) is often used as a tool to objectively and directly measure fatigue (Gerdle et al., 1991; Pincivero et al., 2001) via median power frequency (**MdPF**) and root mean square (**RMS**), which serve as indicators of action potential conduction velocity and muscle fiber recruitment, respectively (Broxterman et al., 2014). Additionally, fatigue in sheep, horses, goats, and cattle have been measured via EMG (Girsch et al., 1995; Robert et al., 2003; Lee et al., 2013; Rajapaksha and Tucker, 2014). If EMG could be adapted for use in swine, fatigued pig syndrome could be studied extensively to help reduce welfare and economic problems in the future. Therefore, the objectives of this literature review are to 1) examine the effects of RAC on finishing performance, skeletal muscle

physiology/biology, and fatigue in swine and 2) determine if EMG characteristics relate to skeletal muscle fatigue.

Chapter 2 - General review of literature

Ractopamine in swine

β -adrenergic receptors and β -adrenergic agonists

β -adrenergic receptors (**BAR**) are found on the membrane of nearly every kind of mammalian cell. The number and type of BAR within each cell varies across species and tissue type. β -adrenergic receptors are composed of a long chain of more than 400 amino acids that form 7 hydrophobic transmembrane domains that secure the receptor to the plasma membrane of the cell. The binding site of the receptor is positioned within the 7 transmembrane domain where the G_s protein is located. When a β -adrenergic agonist (**BAA**) binds to a BAR, the G_s protein is activated allowing for cyclic adenosine monophosphate (**cAMP**) production via adenylyl cyclase fabrication from the G_s subunit. By binding to protein kinase A, cAMP allows for the release of the catalytic subunit that phosphorylates many intracellular proteins. When phosphorylated, these proteins can increase gene transcription including those in skeletal muscle (Strosberg, 1992; Liggett and Raymond, 1993; Mersmann, 1998).

The 3 main types of BAR in mammals are β_1 , β_2 , and β_3 and across species these subtypes differ in distribution and amino acid composition. The homology of amino acid sequence within an individual BAR varies across species and subtypes; however, within a single species, the three receptor types typically have approximately 50% homology in amino acid sequence. Over many species, this homology can present as 75-90% and these differences permit different receptor activation and ligand affinity (Mersmann, 1998; Mills 2002). The number of each receptor type varies across species and tissue type. In swine, β_1 is the most abundant BAR accounting for approximately 73% of all adipose tissue BAR receptors and 59% of all skeletal muscle BAR receptors. The next most abundant BAR in swine are β_2 accounting for

approximately 41% and 21% of adipose and skeletal muscle tissue BAR receptors, respectively. Finally, the β_3 receptor is expressed very little in porcine tissues and composes approximately 7% of all adipose tissue BAR receptors (McNeel and Mersmann, 1995; Liang and Mills, 2002).

Natural BAA are epinephrine and norepinephrine. Epinephrine binds to β_1 and β_2 receptors with the same selective affinity, where norepinephrine has the highest affinity to bind to $\beta_1 > \beta_2 > \beta_3$. Compared to norepinephrine, epinephrine has a greater binding affinity for the β_2 receptor (Mersmann, 1998; Mills 2002). Ractopamine-HCl (**RAC**) is a phenethylamine with BAA properties that simulates epinephrine and norepinephrine. A mixture of 4 stereoisomers (RR, RS, SR, and SS) make up RAC due to the presence of two chiral carbons. Not all of these stereoisomers are biologically active and it is likely that RR is the main functional ligand of RAC; however, the RS stereoisomers can outcompete RR. When the 4 different stereoisomers were administered independently from each other, the RR stereoisomer had the highest binding affinity for both β_1 and β_2 receptors and increased adenylyl cyclase activity compared to the other stereoisomers. The RS stereoisomer had a 15 times lower binding affinity for β_1 and β_2 receptors compared to the RR stereoisomer, but the SS and SR stereoisomers had a 30 times lower binding affinity for β_1 and β_2 receptors compared to the RR and RS stereoisomers. Therefore, with the RR and RS stereoisomers being the most stimulatory, they can often compete with each other to bind to the BAR (Ricke et al., 1999; Mills et al., 2003). Ractopamine is commonly fed during the finishing phase of swine production to ADG, feed efficiency, and muscle growth (Watkins et al., 1990; Moloney et al., 1991). The hypertrophic effect of RAC is due to a combination of increased muscle protein synthesis and decreased muscle protein degradation (Mersmann, 1998). Although this feed additive generally improves finishing performance and carcass characteristics, some variation in responses to RAC have been reported.

Many different factors including dose, time, sex, and weight at initial dose can affect the growth performance and carcass characteristics of pigs fed RAC.

Effects on growth, efficiency and carcass characteristics

Ractopamine level

The dose/response of RAC in the diet has been studied extensively. The manufacturer suggests a level of 5 to 10 mg/kg RAC for the last 20-40 kg of gain (Paulk et al., 2015b). Although the current legal limit of RAC in swine feed is 10 mg/kg RAC, many experiments have been conducted to examine the effect of different RAC levels on performance and carcasses characteristics. Finishing performance in relation to RAC is variable. Some studies support that ADG in pigs increased linearly as the dose of RAC increased (Stites et al., 1991; Crome et al., 1996; Carr et al. 2005; Main et al., 2009; Garbossa et al., 2013), while other publications show that ADG increased by the same magnitude regardless of RAC level (Watkins et al., 1990; Amaral et al., 2009). Armstrong et al. (2004) and Ross et al. (2011) reported no change in ADG when pigs were fed 10 mg/kg RAC. Ractopamine fed at any concentration increased G:F (Barker et al., 2005; Carr et al. 2005; Ross et al., 2011) or decreased feed to gain ratio (**F:G**, Watkins et al., 1990; Stites et al., 1991; Amaral et al., 2009), which are both measures of feed efficiency. There are divergent results on the effect RAC elicits on ADFI. Mimbs et al. (2005) reported that pigs fed 10 mg/kg RAC had decreased ADFI. In contrast, Watkins et al. (1990) reported decreased ADFI when pigs were fed 30 mg/kg RAC, but no change when pigs were fed 5, 10 or 15 mg/kg RAC. Crome et al. (1996) reported that ADFI increased linearly with increasing RAC dose, but many other studies show that regardless of RAC level in the feed, ADFI was not affected (Stites et al., 1991; Barker et al. 2005; Carr et al. 2005; Amaral et al., 2009; Garbossa et al., 2013).

Similar to finishing performance, effects on carcass characteristics are also variable with different inclusion levels of RAC in the diet. At 10 mg/kg RAC inclusion, Paulk et al. (2015a) and Carr et al. (2005) reported increased HCW. Stites et al. (1991) and Crome et al. (1996) showed increased HCW with increasing RAC dose; however, Watkins et al. (1990) reported no difference in HCW regardless of dose. Dressing percent (calculated by $[\text{HCW}/\text{live wt}] * 100$) increased with increasing RAC dose (Stites et al., 1991; Crome et al., 1996). It has also been reported that when pigs were fed 10, 20, and 30 mg/kg RAC, the carcasses had increased dressing percent compared to the carcasses of pigs fed 0 mg/kg RAC (Watkins et al., 1990). Stites et al. (1991) reported increasing loin eye area (**LEA**) in carcass from pigs fed RAC at increasing doses. Watkins et al. (1990) reported that carcasses from pigs fed 2.5, 5, 10, 20, or 30 mg/kg all had larger LEA compared to pigs not fed RAC. Amaral et al. (2009) showed that carcass from pigs fed 10 mg/kg RAC exhibited increased LEA, but this measure was not affected when pigs were fed 5 mg/kg RAC. In contrast to these studies, Mimbs et al. (2005) reported that 10 mg/kg RAC did not affect loin muscle area. Differences between studies could be due to differences in study design such as number of pigs, sex, or housing.

Carcasses from animals fed RAC are typically composed of more muscle and less fat; however, literature analyzing LEA color in response to RAC is variable. Overall backfat was decreased in carcasses from pigs fed 5, 10, 20, and 30 mg/kg RAC (Watkins et al., 1990; Mimbs et al., 2005; Amaral et al., 2009). Crome et al. (1996) showed a linear decrease in 1st rib, 10th rib, and last lumbar backfat thickness with no difference in last rib backfat thickness. When pigs were fed 10 mg/kg RAC, Carr et al. (2005) found no difference in backfat thickness at any location and Stites et al. (1991) reported no difference in 10th rib backfat thickness. Color characteristics of carcasses from pigs supplemented with RAC are not different than pigs not

supplemented with RAC (Watkins et al., 1990; Stites et al., 1991). Marbling scores from RAC fed pigs are extremely variable. Apple et al. (2008) reported increased marbling with 10 mg/kg RAC, Crome et al. (1996) and Carr et al. (2005) showed no change in marbling, and Armstrong et al. (2004) reported a decrease in the amount of marbling with 10 and 20 mg/kg RAC.

Ractopamine feeding duration

Not only does the amount of RAC in the diet affect growth and efficiency, but the time at which pigs are fed RAC also determines the magnitude of the response. Sainz et al. (1993b) fed 40 gilts either 1) 0 mg/kg for 6 wk, 2) 20 mg/kg RAC for 6 wk, 3) 0 mg/kg RAC for 3 wk then 20 mg/kg RAC for 3 wk, 4) 20 mg/kg RAC for 3 wk then 0 mg/kg RAC for 3 wk, or 5) 0 mg/kg RAC for wk 1, 3, 5 and 20 mg/kg RAC for wk 2, 4, 6 of the study. When determining ADG per wk, this study showed that pigs fed RAC for 6 wk had increased ADG in wk 1, 2, and 3 of the feeding period but not in wk 4, 5, and 6. Additionally, the pigs fed 20 mg/kg RAC for 6 wk and the pigs fed 0 mg/kg RAC for wk 1, 3, 5 and then 20 mg/kg RAC for wk 2, 5, 6 of the study had increased total weight gain compared to the other treatment groups. There was a tendency for pigs fed 0 mg/kg RAC for 3 wk and then fed 20 mg/kg RAC for 3 wk to have increased total weight gain compared to all other treatments. There were no differences between groups for carcass weight, backfat, and LEA. According to this study, feeding 20 mg/kg RAC for the final 21 d of finishing is the most effective for performance improvements; however, feeding and of the RAC strategies did not affect carcass characteristics.

Main et al. (2009) used a 3×4 factorial and fed pigs either 0, 5, or 10 mg/kg RAC for either 7, 14, 21, or 28 d. Regardless of RAC dose, treatment period length did not affect ADG, G:F, or carcass yield. Carcasses from pigs fed 5 and 10 mg/kg RAC had decreased fat and increased lean percentage when RAC was fed between d 7 to d 21 on feed. Almeida et al. (2013)

fed 0 or 10 mg/kg RAC for 7, 14, 21, or 28 d. and found a linear increase of ADG and G:F with increasing feeding duration; however, the magnitude of growth after 21 d was not as great and d 7 and d 14. Ractopamine also did not affect backfat measurements, but HCW, dressing percentage, LEA, and muscle to fat ratio all increased linearly with increased feeding duration. Similarly, Gerlemann et al. (2014) found that feeding either 0 or 7.4 mg/kg RAC for 7, 21, or 35 d produced greater ADG in pigs fed RAC regardless of feeding duration; however, the magnitude was decreased as the feeding duration increased. The HCW, carcasses yield, and carcass leanness increased with extended feeding period. These results are different from Sainz et al (1993b), possibly due to the lower RAC dose fed.

Armstrong et al. (2004) also examined the effect of both concentration and duration of feeding on performance and carcass characteristics of finishing pigs. Four-hundred barrows were split into 5 feeding duration treatments: 1) 1 wk, 2) 2 wk, 3) 3 wk, 4) 4 wk, or 5) 5 wk. Within treatment duration, pigs were fed either 1) 0 mg/kg RAC, 2) 5 mg/kg RAC, 3) 10 mg/kg RAC, or 4) 20 mg/kg RAC. In all durations and dose groups, ADG was increased in pigs fed RAC compared to pigs not fed RAC except for pigs fed 5 mg/kg RAC for 4 wk, pigs fed 10 mg/kg RAC for 5 wk, and pigs fed 20 mg/kg RAC for 1 wk. The G:F was increased for all pigs fed RAC compared to pigs not fed RAC; however, pigs fed 20 mg/kg RAC for 3, 4, and 5 wk had a greater magnitude of improvement compared to pigs fed 0 and 5 mg/kg RAC. The HCW was increased in carcass from pigs fed greater doses of RAC for longer durations. This study revealed that increasing dosage for a longer duration did not necessarily improve performance in all aspects, but it did improve carcass characteristics. Although, examining all studies, feeding a lower RAC dose for a longer period could allow for more steady growth rate by not over-loading BAR receptors, which could eventually lead to down-regulation of the BAR receptors.

Step-up feeding programs

Early literature also explored feeding RAC in a step-up program. These studies include increasing the dose of RAC as feeding duration increased. See et al. (2004) fed 200 barrows and gilts either 1) 0 mg/kg RAC for 6 wk, 2) 5 mg/kg RAC for 2 wk then 10 mg/kg RAC for 2 wk, then 20 mg/kg RAC for 2 wk 3) 20 mg/kg RAC for 2 wk, then 10 mg/kg RAC for 2 wk, then 5 mg/kg RAC for 2 wk, or 4) 11.7 mg/kg RAC for 6 wk. Regardless of the feeding program, all pigs fed RAC had increased ADG and G:F by the same magnitude. Carcasses from pigs fed a step-up and constant RAC feeding program had increased HCW and percent yield compared to carcasses from pigs fed 0 mg/kg RAC or pigs fed a step-down program. Armstrong et al. (2005) divided 1,050 pigs into 4 treatment groups: 1) 0 mg/kg RAC for 35 d, 2) 5 mg/kg RAC for 35 d, 3) 5 mg/kg RAC for 14 d then 10 mg/kg RAC for 21 d, or 4) 5 mg/kg RAC for 21 d then 10 mg/kg RAC for 14 d. Compared to pigs fed 0 mg/kg RAC and pigs fed a constant RAC concentration, pigs fed a step-up program had increased ADG and feed efficiency. The ADG was not different between any treatment groups and carcasses from pigs fed a step-up program tended to be heavier compared to carcasses from pigs fed a constant RAC diet. The percent yield was increased in pigs fed RAC compared to those not fed RAC, however the magnitude of improvement was similar between all RAC treatments.

Poletto et al. (2009) fed 64 barrows and gilts either 1) 0 mg/kg RAC for 28 d or 2) 5 mg/kg RAC for 14 d then 10 mg/kg RAC for 14 d. There were no differences in ADG and G:F between treatments in the first 14 d of feeding; however, at d 28, ADG and G:F were increased in the pigs fed step-up RAC compared to the pigs fed 0 mg/kg RAC. The LEA and backfat measurements were not different between treatment groups. Similarly, Bohrer et al. (2013) fed 200 gilts and barrows either 1) 0 mg/kg RAC for 28 d or 2) 7.4 mg/kg RAC for 14 d then 10 mg/kg RAC for 14 d. The ADG and G:F were increased in pigs fed step-up RAC compared to

pigs fed 0 mg/kg RAC and the ADFI was not different between groups. The LEA was increased in the pigs fed step-up RAC, but the 10th rib backfat was not different between groups. The major flaw in Poletto et al. (2009) and Bohrer et al. (2013) studies is that there was no treatment group that was fed a constant RAC diet throughout the feeding period. Studies were conducted on step-up RAC feeding programs because long-term, high-dose RAC feeding has been linked to down-regulation of BAR receptors, which can limit the maximum growth and carcass characteristics (Williams et al., 1994); however, when concluding step-up RAC feeding studies, pigs fed a step-up RAC diet do not seem to have a large advantage over pigs fed a constant RAC diet when examining growth performance and carcass characteristics.

Ad libitum vs restricted feeding

Another factor to consider when feeding RAC, is whether or not pigs have *ad-libitum* or restricted access to feed. Smith et al. (1995) fed pigs either a restricted or *ad-libitum* diet containing 1) 0 mg/kg RAC, 2) 5 mg/kg RAC, 3) 12.5 mg/kg RAC, or 4) 20 mg/kg RAC. Pigs fed RAC on the restricted diet had increased ADG compared to pigs fed restricted 0 mg/kg RAC; however, the ADG of pigs fed RAC *ad-libitum* were not different compared to pigs fed *ad-libitum* with 0 mg/kg RAC. The LEA was increased in carcasses from pigs fed restricted and *ad-libitum* RAC compared to pigs fed 0 mg/kg RAC in both groups. In contrast, Cantarelli et al. (2009) found the feeding RAC in *ad-libitum* and restricted diets resulted in the same improvement in ADG and feed efficiency for pigs fed RAC compared to pigs not fed RAC, but there were no carcass characteristics reported. In conclusion, more research is needed to determine if restricted or *ad-libitum* feeding of RAC results in improved growth and carcass characteristics in swine.

Starting weight

The weight at which pigs are initially fed RAC during the finishing phase could affect growth and carcass characteristics. Robinson (1976) hypothesized that as body weight increases, lean growth weight declines, and additionally, lean growth rate reaches its plateau at approximately 70-90 kg. This plateau of lean growth would occur midway through the exponential growth phase, meaning that the remainder of the exponential growth phase would include fat growth. Gu et al. (1991b) fed barrows either 0 mg/kg RAC or 20 mg/kg RAC during 3 different growth phases: 1) 59-100 kg, 2) 73-114 kg, or 3) 86-127 kg. This study reported growth performance traits and revealed increased average daily lean gain, total lean gain, and lean efficiency in all pigs fed RAC, and pigs in the 73-114 kg weight group had the most lean growth. Though this is important information, these authors never analyzed a RAC \times Weight phase interaction, which is a serious flaw in the study design. In another study conducted at the same institution, Gu et al. (1991a) used the same study design and analyzed the RAC \times Weight interaction, but reported only the carcass characteristics. The authors found that RAC increased the dressing percent and last lumber backfat in carcasses from barrows in the 73-114 kg weight group compared to the other weight periods. Additionally, Crome et al. (1996) fed barrows and gilts either 1) 0 mg/kg RAC, 2) 10 mg/kg RAC or 3) 20 mg/kg RAC during 2 different growth phases: 1) 68-107 kg, or 2) 85-125 kg. There were no RAC \times Weight group interactions for ADG, HCW, LEA, marbling, or dressing percent; however, RAC decreased ADFI, F:G, and 10th rib backfat in carcasses from pigs from the 85-125 kg group. The LEA area was not reported, but typically decreased 10th rib backfat is an improvement coupled with increased LEA. Though there are few studies that examine feeding RAC during different weight stages, it could be concluded that starting pigs on RAC when they are around 70-85 kg can be the most beneficial for growth and carcass characteristics.

Castration and Sex

Few studies examine the effect of RAC and castration on growth and efficiency in swine. Braña et al. (2013) used a 3×2 factorial of 1) gender (gilt, surgically castrated barrow [SC], or immunologically castrated barrow [IC]) and 2) RAC level (0 or 5.5 mg/kg RAC for 28 d) to examine RAC and castration method/sex on growth performance. There were no RAC \times Sex interactions for ADG or G:F anytime during the feeding period; however, there was a RAC \times Sex interaction for ADFI. Gilts fed 0 mg/kg RAC had decreased ADFI compared to the IC and PC barrows fed 0 mg/kg RAC. There were also no RAC \times Sex interactions for HCW, carcass yield, backfat, or LEA. Similarly, Puls et al. (2014) used a 3×3 factorial consisting of 1) sex (gilt, IC barrow, and PC barrow) and 2) RAC inclusion level (0, 5, or 7 mg/kg RAC for 26 d). There were no RAC \times Sex interaction for ADG, G:F, ADFI, HCW, or carcass yield. Lowe et al. (2014) also found no RAC \times Castration method interactions for ADG, G:F, or ADFI when feeding IC or PC barrows 0 or 5 mg/kg RAC for 33 d. In general, barrows and gilts have similar growth responses to RAC regardless of castration method.

Other studies focus on sex (regardless of castration method) and RAC on growth performance. Dunshea et al. (1993b) used 20 gilts, 20 barrows, and 20 boars in a feeding experiment. Pigs from each sex were fed either 0 mg/kg RAC or 20 mg/kg RAC during the finishing phase. There were RAC \times Sex interactions for ADG and F:G. The ADG was increased in gilts and barrows fed RAC compared the boars fed RAC. Similarly, the gilts and barrows had decreased F:G compared to the boars. There were no RAC \times Sex interactions for ADFI, backfat, LEA, or HCW. In the same year, Uttaro et al. (1993) conducted a similar study feeding gilts and barrows either 0 or 20 mg/kg RAC. These authors did not analyze a RAC \times Sex interaction; however, when pooled together both gilts and barrows had increased ADG and decreased F:G and 10th rib backfat when fed RAC. Smith et al. (1995) fed gilts and boars either 0, 5, 12.5, or 20

mg/kg RAC and the ADG was not different between sexes at any RAC level, but the authors did not analyze ADFI for comparison between sexes. The LEA increased linearly and the total loin backfat percent decreased linearly with increasing dose of RAC in gilts but not barrows. Poletto et al. (2009) examined the effects of RAC and sex on growth characteristics when animals were exposed to a step-up program. Sixty-four barrows and gilts were blocked by sex and exposed to 1 of 2 treatments: 1) 0 mg/kg RAC for 28 d or 2) 5 mg/kg RAC for 14 d followed by 10 mg/kg RAC for 14 d. This study resulted in no RAC \times Sex interactions for ADG, G:F, or ADFI at any point during the diet treatment period. Initially, at the beginning of the feeding period, barrows had increased back fat at the 10th and last rib compared to gilts; however, at the completion of feeding, there was no difference between sexes. Amaral et al. (2009) also found no RAC \times Sex interaction for ADG, F:G, ADFI, carcass yield, backfat thickness, or LEA when gilts and barrows were fed either 0, 5, or 10 mg/kg for 28 d. According to the literature above, barrows and gilts have similar responses to RAC, whereas boars do not seem to benefit as much as other sexes. This could be due to the increased testosterone level in boars allowing them to grow faster and deposit more lean growth compared to gilts and barrows.

Effects on fiber cross sectional area and isoform distribution

Adult swine have four skeletal muscle fiber types including type I, IIA, IIX, and IIB. These fiber types can be classified based on multiple characteristics including molecular, metabolic, structural, and contractile properties. One way to identify fiber types is based on their myosin isoform profile, which are measures of both structural and functional properties. Fibers are composed of four major myosin heavy chain (**MyHC**) isoforms: MyHC I, MyHC IIA, MyHC IIX, and MyHC IIB respectively (Schiaffino and Reggiani, 1994). Contractile speed, myosin isoform, and energy are factors used to classify muscle fibers. Type I fibers are slow

contracting and highly oxidative. This allows for endurance type activities due to having fatigue resistant properties. Type IIB are fast contracting and highly glycolytic, meaning they are the most easily fatigable fiber type. Type IIA and IIX are intermediate fibers, varying in glycolytic metabolism rate, with type IIX having greater contractile speed and decreased oxidative rate than type IIA (Pette and Staron, 2001). Introducing a BAA compound into livestock diets change skeletal muscle properties by increasing lactate dehydrogenase activity and decreasing oxidative enzymes. This increases glycolysis since glucose is being metabolized to lactate, which results in ATP being produced at the substrate level instead of ATP being produced via oxidative phosphorylation (Eisemann et al., 1988; Vestergaard et al., 1994). This can change the size, frequency, proportion, and isoform distribution of muscle fibers. This, in turn, can alter the muscular make up of an animal and allow for different muscle contractile properties.

There are many techniques that can be used to determine muscle fiber type or muscle MyHC isoform identification. Histology that can be used include adenosine triphosphate (**ATPase**) stains that identify fiber speed, succinate dehydrogenase (**SDH**) stains that identify fiber oxidative properties, and immunofluorescent stains that identify myosin heavy chain proteins. Myosin protein analysis can be done by western blot fluorescent staining. Additionally, myosin isoform gene expression can be measured by polymerase chain reaction (**PCR**).

Adenosine triphosphate stains detect enzymes that impact muscle fiber contraction speed. Adenosine triphosphates are enzymes that catalyze adenosine triphosphate (**ATP**) to adenosine diphosphate (**ADP**) + P_i after a muscle contraction (Geider and Hoffmann-Berling, 1981). For this type of staining, muscle sections are pre-incubated in either an acidic or alkaline solution. Acidic pre-incubations inactivate fast myosin ATPase activity (in type II muscle fibers) and alkaline pre-incubation solutions inactivate slow myosin ATPase activity (in type I muscle

fibers). During the staining procedure, P_i reacts with calcium (**Ca**) to form a calcium phosphate (**CaPO₄**) precipitate. This is then converted into cobalt sulfide (**CoS₂**) that is brownish/black in color. As ATP is used, P_i is released leaving the brown/black color on the tissue section (Brooke and Kaiser, 1970). If tissue samples are pre-incubated in an acidic solution, type I fibers will be dark and type II fibers will stain lighter. Conversely, if tissue samples are pre-incubated in alkaline solution, the type II fibers will stain darker and type I fibers will be lighter in color (Behan et al., 2002).

Succinate dehydrogenase stains detect different fiber types based on oxidative properties. Succinate dehydrogenase is found in the inner membrane of the mitochondria, where the ability to produce ATP is dependent on oxidative phosphorylation. During the staining procedure, tissues incubate in a solution containing nitroblue tetrazolium (**NBT**), sodium succinate, and a phosphate buffer. As the tissue incubates, succinate converts to fumarate which chemically reacts with NBT resulting in the mitochondria staining purple. The darker fibers represent more oxidative cells whereas the lighter fibers represent more glycolytic cells (Kern et al., 1999).

Immunofluorescent stains are becoming more common. This type of stain uses primary and secondary antibodies to express different fiber types. Tissue sections incubate in a blocking solution to prevent non-specific binding (meaning the antibodies used could bind to things other than the intended antigen), then incubate in primary antibodies that bind to specific proteins, and then incubate in secondary antibodies which bind to the primary antibodies allowing for fluorescence. Using immunofluorescent staining allows for the complete differentiation of hybrid fibers including type I, IIA, and IIB fibers and non-hybrid fibers including type IIX fibers (Kammoun et al., 2014) much easier than ATPase or SDH stains.

Aalhus et al. (1992) fed 52 barrows and gilts either 0 mg/kg RAC or 20 mg/kg RAC. After harvest, the *Semimembranosus* and the *Psoas major* muscles were taken for fiber type distribution and cross sectional area (CSA) analysis by myosin ATPase stain. The frequency of fiber types (red [type I], intermediate [type IIA and IIX], or white [type IIB]) were determined by counting 1 to 3 muscle bundles per sample (75 fibers per sample). For each fiber type, the minimum and maximum diameters were measured within a bundle (15 fibers per sample) and the CSA was determined by the formula created by Clancy and Herlihy (1978):

$$\frac{\pi}{4} D \cdot d$$

Where D = maximum diameter and d = minimum diameter. Pigs fed 20 mg/kg RAC showed no change in the percentage of red fibers, decreased intermediate fibers, and increased white fibers in the *Semimembranosus* and *Psoas major* muscles compared to pigs fed 0 mg/kg RAC. The CSA of the *Semimembranosus* and *Psoas major* did not change in the red fibers and increased in the intermediate and white fibers in the pigs fed 20 mg/kg RAC compared to pigs fed 0 mg/kg RAC. This study suggests that RAC causes muscle fibers to shift towards glycolytic metabolism at the expense of intermediate fibers and that RAC causes intermediate and white fibers to increase in size.

Three years later, Sainz et al. (1993a) also studied whether RAC produced changes in muscle fiber type distribution and CSA. Forty gilts were split into 5 treatment groups: 1) fed a diet containing 0 mg/kg RAC for 6 wk, 2) fed a diet containing 20 mg/kg RAC for 6 wk, 3) fed diet containing 0 mg/kg RAC for 3 wk then 20 mg/kg RAC for 3 wk, 4) fed a diet containing 20 mg/kg RAC for 3 wk then fed a diet containing 0 mg/kg RAC for 3 wk, or 5) fed a diet containing 0 mg/kg RAC for wk 1, 3, and 5 and then fed a diet containing 20 mg/kg RAC for wk 2, 4, and 6 of the study. After the conclusion of feeding, gilts were harvested and samples from the *Longissimus*

dorsi were taken from 5 gilts per treatment for ATPase histology staining. The authors were able to identify type I, type II, and giant fiber (which were identified by being well-rounded and larger than 6,000 μm^2). Ractopamine did not affect fiber percentage between treatments. The CSA of type I fibers were not different between treatments. Type II fibers had smaller CSA in the group fed 0 mg/kg RAC for 3 wk and then 0 mg/kg RAC for 3 wk compared to the group fed 0 mg/kg RAC for 6 wk. Type II fibers and giant fibers had larger CSA in the group fed 0 mg/kg RAC for 3 wk and then 20 mg/kg RAC for 3 wk compared to the group fed 0 mg/kg RAC for 6 wk. This study showed no distribution differences between treatments; however, the pigs fed RAC later in the finishing phase produced larger fibers similar to Aalhus et al. (1992).

Interestingly, Sainz et al. (1993a) did not find differences in fiber CSA size or distribution between the control group and the group fed RAC for 6 wk. It is possible that the pigs fed RAC for 6 wk maxed out their growth potential, plateaued, and then declined making RAC administration ineffective after the plateau phase. Other studies have found this to be the case (Spurlock et al., 1994; Williams et al., 1994). This could be due to decreased cellular responses of increased protein deposition and decreased fat deposition as the time on RAC increases (Williams et al., 1994). Williams et al. (1994) also suggests that the maximum increase in growth occurs during a rapid growth stage of development and when the growth potential decreases, so does the effect of RAC. It is also possible that the decrease in growth is due to the down-regulation of β -receptors (Dunshea et al., 1993a).

Depreux et al. (2002) took fiber distribution one step further and specifically targeted all 4 swine fiber types. Sixty pigs were split into 3 treatments: 1) fed 0 mg/kg RAC, 2) fed 20 mg/kg RAC, or 3) fed 60 mg/kg RAC. After the start of treatment diets, 4 animals per treatment were harvested on d 3, 7, 14, 28, and 42 and samples from the *Longissimus* and parts of the red

Semitendinosus (deep portion of muscle) and white *Semitendinosus* (superficial portion of muscle) muscles were taken for myosin protein analysis. The myosin proteins were extracted and the amount of muscle MyHC isoform was determined by using an ELISA kit created by Depreux et al. (2000). The different isoforms were identified by using antibodies against MyHC type I, IIA, IIAX (type IIX), and IIB.

Overall harvest times, MyHC I decreased in the red *Semitendinosus* and white *Semitendinosus*, but remained the same in the *Longissimus* as the RAC dose increased. Across all muscles, pigs fed 60 mg/kg RAC had decreased amounts of MyHC IIA and IIAX and increased amounts of MyHC IIB at d 42 vs d 3 of feeding. At d 42, the abundance of type IIA and IIAX MyHC was decreased in pigs fed 20 mg/kg RAC compared to pigs fed 0 mg/kg RAC, but did not differ between pigs fed 20 mg/kg RAC compared to pigs fed 60 mg/kg RAC. Pigs fed 60 mg/kg RAC had decreased MyHC IIA and IIAX compared to pigs fed 0 mg/kg. The abundance of type IIB MyHC at d 42 was increased in pigs fed 20 mg/kg RAC compared to pigs fed 0 mg/kg RAC, in pigs fed 60 mg/kg RAC compared to pigs fed 20 mg/kg RAC, and in pigs fed 60 mg/kg RAC compared to pigs fed 0 mg/kg RAC. This study suggest that with increased time and dose of RAC, the MyHC isoforms transition from more oxidative type II protein (MyHC IIA and IIX) towards glycolytic protein (MyHC IIB).

Gunawan et al. (2007) used PCR to measure MyHC gene expression in 2 different experiments. In experiment 1, 44 pigs were fed 20 mg/kg RAC for 0, 1, 2, or 4 wk. After 4 wk, pigs were harvested and samples were taken from the *Longissimus* muscle for PCR analysis. Relative transcript abundance was determined by using specific primer sequences for MyHC isoforms I, IIA, IIX, IIB, and total MyHC. Expression of type I MyHC was not different between treatments. Myosin heavy chain IIA was decreased in pigs fed RAC for 1wk, decreased further in

pigs fed RAC for 2 wk, but was not different in pigs fed RAC for 4 wk compared to pigs fed RAC for 0 wk. The expression of MyHC IIX was not different in pigs fed RAC for 1 wk compared to pigs fed RAC for 0 wk; however, pigs fed RAC for 2 and 4 wk had decreased expression compared to pigs fed RAC for 0 wk. The expression of MyHC IIB was increased in pigs fed RAC for 1, 2, and 4 wk compared to controls. There was increased expression of total MyHC in pigs fed RAC for 1 and 2 wk compared to pigs fed RAC for 0 wk; however, there was no difference in pigs fed RAC for 4 wk compared to pigs fed RAC for 0 wk.

In experiment 2, 48 pigs were split into 2 treatment groups 1) 0 mg/kg RAC or 2) 20 mg/kg RAC. Six pigs from each treatment group were harvested after 12, 24, 48, or 96 h and samples of the *Longissimus*, red *Semitendinosus* and white *Semitendinosus* were taken for the same PCR analysis as experiment 1. In all muscles, the MyHC IIA expression was decreased at 96 h in pigs fed 20 mg/kg RAC compared to pigs fed 0 mg/kg RAC. In all muscles, the expression the MyHC IIB was increased at 12, 24, 48, and 96 h in pigs fed 20 mg/kg RAC compared to pigs fed 0 mg/kg RAC. There was no Muscle \times Time interaction for MyHC gene expression. Similar to previous studies, RAC did not affect slow fiber type (type I) expression, but it did cause shifts towards more type II glycolytic expression (type IIB).

To depict the effect of RAC on muscle fiber types in swine using immunohistochemical staining, Paulk et al. (2014) fed 80 finishing pigs 0 mg/kg RAC or 10 mg/kg RAC for 35-40 d before harvest. At harvest, samples of the *Longissimus lumborum* were taken from 20 pigs per treatment for fiber type distribution analysis. The percentage of type I and IIA fibers were not different between treatment groups. Type IIX fiber percentage was decreased in the 10 mg/kg RAC group compared to the 0 mg/kg RAC group; however, there was a tendency for type IIB fibers to be increased in the 10 mg/kg RAC group compared to the 0 mg/kg RAC group. This study showed

that oxidative fiber types are not affected by RAC, but fibers shift towards type IIB at the expense of type IIX fibers. Burnett et al. (2016) evaluated the CSA of the same group of pigs and reported that the Type I and IIB fiber CSA were not affected by RAC, but RAC pigs tended to have larger type IIX fibers than CON pigs, and the type IIA fibers were larger in the pigs fed RAC.

Research targeting the effect of muscle fiber size, proportion, and MyHC expression in response to BAA suggests that only type II fibers are affected. Furthermore, type IIB fibers seem to benefit at the expense of the intermediate type II fibers (IIA and IIX) in size and proportion. Few studies emphasize the effect of RAC on muscle fiber type in swine and different methods are used in all of them; however, these studies show that RAC does not only improve lean muscle growth, but it also changes muscle metabolism at a cellular level. These changes could alter meat quality and muscle fatigability. Since size of muscle fibers share an inverse relationship with oxidative properties (van Wessel et al., 2010), animals fed RAC should experience fatigue quicker since their muscle fibers change to contain more glycolytic properties due to the increased size and proportion of type IIB fibers. Since fast-twitch muscle fibers rely on substrate level phosphorylation, the ATP production is limited resulting in a faster fatigue rate (Kang, 2013).

Effects on succinate dehydrogenase

Succinate dehydrogenase is an enzyme present in all aerobic cells and is part of the electron transport chain and Krebs cycle. Several activators and inhibitors modulate its activity, and it contains a nonheme iron, acid-labile sulfur, covalently bound flavin adenine dinucleotide (**FAD**). Succinate dehydrogenase catalyzes the oxidation of succinate to fumarate and transfers the resulting reducing equivalents directly to the electron transport chain (Hederstedt and

Rutberg, 1981). Succinate dehydrogenase is found in the inner membrane of the mitochondria, where the ability to produce ATP is dependent on oxidative phosphorylation (Kern et al., 1999).

No studies to date have examined the effects of RAC or any BAA compound on SDH in swine, cattle, or sheep and relatively few studies exist in rodents. Lynch et al. (1996) administered either 0 or 2mg/kg clenbuterol (BAA) in the drinking water of male mice for 15 w. With the use of a SDH staining procedure, it was reported that mice administered the BAA had decreased SDH in the *Extensor digitorum longus* and *Soleus* muscles. Similarly by using SDH staining techniques, Suzuki et al. (1997) fed young and middle aged rats either 0 or 2 mg/kg clenbuterol for 10 d and reported decreased SDH in the *Soleus*, *Plantaris*, and deep and superficial *Gastrocnemius* muscles. Although there are very few studies examining effects of BAA on SDH activity, the literature suggests that the introduction of a BAA causes a decrease in SDH, which would result in less oxidative metabolism in skeletal muscle.

Effects on blood flow

Primary arteries are the last segments of arterial blood supply before entering skeletal muscle tissue. These arteries are located along the axis of muscle tissue and connect to arteries that angle toward the epimysium of the muscle. From the epimysium, secondary arteriolar branches divide into longitudinal feed vessels that further branch into arterioles that enter the perimysium and run perpendicular to the muscle fiber axis. Once these arrive at the endomysium, they are embedded and run parallel to each muscle fiber and become capillaries. The capillaries are the smallest functional unit for blood flow to skeletal muscle tissues and they surround muscle fibers in a non-uniform distribution that allows for non-homogeneous distribution of oxygen to muscles (Berg and Sarelius, 1995; Bagher and Segal, 2011). Capillaries are important for exchange of nutrients and waste in the muscle fiber (Aberle et al., 2003). Approximately 4

µm in size, all capillaries within muscle create a network allowing for blood perfusion.

Additionally, oxidative fibers contain a greater capillary supply than glycolytic fibers (Folkow and Halicka, 1968; Andersen, 1975; Hudlicka, 1985).

The use of BAA in livestock can increase blood flow, especially to skeletal muscle, where energy sources and substrates are delivered for protein synthesis to increase muscle hypertrophy. The increase in blood flow is likely due to increased blood pressure. Additionally, it is hypothesized that increased blood flow to adipose tissue removes nonesterified fatty acids, allowing for enriched lipid degradation (Mersmann, 1998). Mersmann (1989) infused anesthetized pigs with increasing concentrations of BAA (norepinephrine + epinephrine + dopamine, isoproterenol, or clenbuterol) and found, by collecting heart rate and blood pressure data, that there was increased blood flow to skeletal muscles with increased BAA level administration. Eisemann et al. (1988) reported that cattle fed clenbuterol for 1 and 9 d had increased heart rate and blood flow to the hind-quarters after administration. The increased blood flow in response to BAA could be due to increased heart rate, which has been reported in swine (Mersmann, 1987). Marchant-Forde (2003) reported pigs fed 10 mg/kg RAC for 28 d had increased heart rates when exposed to non-familiar humans; however, James et al. (2013) reported that there was no difference in heart rate after being subjected to a simulated transportation model when pigs were fed either 0 or 20 mg/kg RAC for 28 d.

Although blood flow and heart rate have been examined in swine administered BAA, capillary density in response to administration has not. Vastergaard et al. (1994) reported that bulls administered cimaterol for 90 d had larger muscle fibers, but no change in the capillary to fiber ratio. Although the capillary to fiber ratio did not change, the BAA decreased the number of capillaries per square mm in the *Longissimus* and *Semitendinosus* muscles. Suzuki et al. (1997)

fed young and middle aged rats either 0 or 2 mg/kg clenbuterol and reported no capillary to fiber ratio difference between diet groups in the *Soleus*, *Plantaris*, and deep and superficial *Gastrocnemius* muscles at any level of the capillary bed.

Muscle fatigue in swine

How muscles fatigue

Skeletal muscle is attached to bones either directly, or indirectly by ligaments, cartilage, fascia, or skin. The structure of muscle is very complex. The whole muscle is surrounded in a covering called the epimysium. Within the whole muscle there are groups of muscle bundles containing muscle fibers. The muscle bundles are surrounded by a covering called the perimysium. The muscle fiber is the cellular unit of muscle. It is surrounded by the sarcolemma, which is where the motor neuron end plate terminates at the myoneural junction. The sarcolemma is made of a protein lipid material that allows for flexibility during contraction. The endomysium, which is composed of fine collagen fibers, covers the sarcolemma. Cavities formed in the sarcolemma and around muscle fibers create a network of T-tubules that allow for action potentials to travel from the surface of the cell to inside to cell in order to open calcium channels on the sarcoplasmic reticulum. Muscle fibers contain 1,000 to 2,000 myofibrils each and each myofibril contains approximately 10,000 sarcomeres. Myofilaments including actin (thin filaments) and myosin (thick filaments) are the contractile proteins of muscle (Aberle et al., 2003).

Four main myofibrillar proteins are involved in muscle contraction: actin, myosin, tropomyosin, and troponin. Troponin has 3 subunits, I, C, and T. Actin, contains binding sites for myosin heads that are covered by tropomyosin in a resting state. This occurs because troponin-T is bound to tropomyosin while troponin-I is interacting with actin to inhibit a cross-bridge

formation between actin and myosin. At rest, the myosin heads are bound to ADP•Pi and the heads are positioned at a 90° angle. In order for a muscle contraction to occur, an action potential travels down a motor neuron to the motor end plate where acetylcholine is released into the synaptic cleft. Acetylcholine then binds to ligand-gated sodium channels in the synaptic troughs allowing for the action potential to travel down the sarcolemma, and through the T-tubule into the sarcoplasmic reticulum, where voltage-gated calcium channels are opened causing calcium to rush out into the sarcoplasm and bind to troponin-C. This inactivates troponin-I and troponin-T, which causes tropomyosin to rotate, revealing myosin binding sites on actin. The myosin head binds to actin, releases Pi, which causes myosin to rotate to a 45° angle creating a power stroke for contraction (cross-bridge formation). In order for the contraction to be broken, ATP binds to the myosin head causing actin and ADP to be released. Myofibrillar actomyosin adenosine triphosphatase (**mATPase**) then degrades ATP to ADP•Pi and the myosin rotates back to a 90° angle of the relaxed state. Tropomyosin rotates back over myosin binding sites on actin. When ATP is no longer present to relax muscle contraction, muscle fatigue occurs (Aberle et al., 2003).

Adenosine triphosphate is produced by glycolysis via substrate level phosphorylation and the Krebs cycle via oxidative phosphorylation. In order to complete glycolysis and the Krebs cycle, glucose is required. Glucose is stored in the liver and skeletal muscle as glycogen. Muscle glycogen is responsible for approximately 75% of total body glycogen stores. When muscles contract for long periods of time or produce more contractions due to increased physical demands, muscle metabolism shifts from aerobic to anaerobic metabolism which produces less ATP. This occurs due to decreased oxygen supply in the muscle fibers as the oxygen is continuously being used to fuel the Krebs cycle. Additionally, this is directly tied to muscle fiber type since the oxidative fibers are able to utilize oxygen for a greater period of time compared to the glycolytic

fibers (Bender and Mayes, 2006). When considering muscle fatigue in swine, decreased ATP production is very important.

Another factor contributing to fatigue is the skeletal muscle type. Type I fibers are slow contracting and are highly oxidative, which allows for prolonged endurance type activities because of the fatigue resistant properties. Type IIB are fast contracting and highly glycolytic, allowing for short, high intensity activities. Type IIA and IIX are intermediate fibers varying in glycolytic metabolism rate and contractile speed. Type IIX fibers contract faster and have less mitochondria than type IIA. Supportive muscles contain more slow-twitch fibers, while locomotor muscles contain more fast-twitch fibers (Pette and Staron, 2001). The physical ability of animals is dependent on the skeletal muscle fiber type composition throughout the body.

Enoka and Stuart (1992) describe muscle fatigue as a cascade of events that impair motor performance. These authors suggest 4 theories that explain muscle fatigue by neural and neuromuscular mechanisms. The first theory is task dependency, meaning that the rate of fatigue is dependent on the task performed, which also includes the level of motivation and the intensity and duration of the task. The mechanisms that are altered due to various tasks include central nervous system drive (central motor drive) to motor neurons; the muscles and motor units; neuromuscular propagation excitation-contraction coupling; metabolic substrate availability; and muscle blood flow. The second theory is the force-fatigability relationship, meaning, the greater force exerted by a muscle or motor unit, the quicker the muscle will fatigue. This relationship suggests that the mechanisms underlying a shift towards fatigue in a specific muscle with physical activity is dependent on the amount of force exert on that muscle. The authors coined the term muscle wisdom for the third theory. This means that as muscles experience sustained activity, adaptations occur that involve a concurrent decline in force, relaxation rate, and motor unit firing

rate that change over time with increased physical activity or training. This results in an economical activation of muscle fatigue by the central nervous system by optimizing the total muscle force while using minimum resources. Lastly, the fourth theory is the sense of effort associated with a task. The amount of effort is detected by the central nervous system which responds with motor commands. When a specific task is perceived as difficult, the subject will fatigue quicker than if the task is perceived as easy.

Blood metabolites: glucose, Lactate, glutamate, and glutamine

The lactate threshold is defined as the physical activity intensity that is associated with a substantial sustained increase in blood lactate. Additionally, when oxygen availability is limited, lactic acid is formed (Svedahl and MacIntosh, 2003). More lactate is produced when type IIX and type IIB motor units are activated since these usually use ATP produced via glycolysis. Additionally, glycolytic fibers produce lactate at such a rapid pace that the body is unable to clear it as fast as it is being produced. Plasma lactate concentrations remain close to resting levels with increasing levels of exercise until the lactate threshold is reached and muscle fatigue usually occurs shortly after this happens (Achten and Jeukendrup, 2004; Garnacho-Castaño et al., 2015). Although the trend of increasing lactate with increasing work rate is common among subjects during physical activity, the metabolic rate at which the lactate threshold is reached varies between subjects due to different training and fitness levels. This threshold also varies depending on the physical activity task. For example, lactate elimination can occur faster than lactate production if the exercise protocol contains breaks in physical activity, causing the lactate threshold to occur later in the exercise procedure (Heck et al., 1985).

Feedforward and feedback mechanisms are used when controlling glucose regulation during increased physical activity (Suh et al., 2007). In humans, plasma glucose levels elevate

with exercise due to increased hepatic glucose production to meet the amplified demand of glucose uptake in active muscle fibers (Kj  r, 1988), which is the result of a feedforward loop. Glucose production during the beginning of physical activity is mostly from glycogenolysis, but gluconeogenesis also contributes approximately 10-20% total glucose production from the liver. As physical activity increases, gluconeogenesis increases approximately 50% of glucose production. This rise occurs simultaneously with the decrease in liver glycogen stores as they are being used. If plasma glucose levels fall during physical activity, the alpha cells in the pancreas release the hormone glucagon, which gets converted into glucose in the liver, and released into the blood creating a feedback loop (Suh et al., 2007). Weiss et al. (1974) gave pigs from a stress-susceptible or fat line one of two alpha blockers (to decrease blood pressure) or no drug at all. After 5 min of increased physical activity, both plasma glucose and plasma lactate were increased regardless of treatment or genetic line.

Glucose is not the only metabolite important for cellular metabolism. The amino acids glutamate and glutamine are important for protein synthesis and muscle growth. Glutamine is able to regulate gene expression and mitogen-activated protein kinase activation, and glutamate is an important anaplerotic precursor for the Krebs cycle, meaning it helps replenish the depleted metabolites needed for the Krebs cycle (Gibala et al., 1997; Bowtell and Bruce, 2002; Newsholme et al., 2003). Increased glutamate and decreased plasma glutamine after prolonged exercise in humans are often indicators of muscle damage (Keast et al., 1995; Walsh et al., 1998; Leibowitz et al., 2012). Hackl et al. (2009) reported decreased glutamine and increased glutamate after an intense bout of exercise in trotting horses.

Fatigued pig syndrome and transport losses

In 2005, Ritter et al. (2005) reported that greater than 50% of non-ambulatory pigs at the packing plant are fatigued and approximately 0.3% of all pigs transported develop fatigued pig syndrome. Fatigued pig syndrome describes non-ambulatory, non-injured pigs without obvious sign of trauma. This occurs anytime from loading at the farm to stunning at the packing plant. To describe the peripheral model of fatigue, Lambert et al (2005) explained that fatigue is a result of substrate depletion and/or metabolic accumulation. The authors suggest that the way to avoid fatigue is to reduce the workload or stop physical activity. Both substrate depletion and metabolic accumulation could occur during fatigued pig syndrome. Pigs are taken off feed prior to transport, which could result in less circulating glucose, contributing to substrate depletion. Additionally, as swine experience fatigue during transport, anaerobic metabolism can create metabolic accumulation, including lactate production. Additionally, pigs are selected for rapid muscle growth, and normally, heavier muscled pigs have increased muscle metabolic rates (Ruusunen and Puolanne, 1997). Increased metabolic rate can lead to increased metabolic accumulation, suggesting a faster rate of fatigue.

Open mouth breathing is often a behavioral sign of fatigue in swine, as it denotes increased demand of oxygen. Muscle tremors, reluctance to move, reddened skin, and increased body temperature are also signs of fatigue in pigs (Anderson et al., 2002). When pigs were loaded into a trailer with an electric prod or a plastic hurdle, pigs subjected to the electric prod had increased heart rate and body temperature (Brundige et al., 1998). Additionally, D'Souza et al. (1998) used 48 pigs in a pre-slaughter handling study where pigs were either handled with or without an electric prod before harvest. Pigs that were subjected to an electric prod had decreased glycogen content in the *Longissimus thoracis* and *Biceps femoris* after harvest. Benjamin et al. (2001) created a model to fatigue pigs by running them 100 m down a narrow alley with unpredictable,

abrupt handling techniques including electric prods. More than 20% of pigs in this study were fatigued by this model. This conclusion was made based on increased skin discoloration, vocalization, and breathing.

Ritter et al. (2007) examined the effect of distance moved during loading (short: 0-30.5 m or long: 61-91.4 m) and the floor space per pig during transport (0.396, 0.415, 0.437, 0.462, 0.489, or 0.520 m²/pig) on transport losses. Pigs that were moved a longer distance during loading displayed more open mouth breathing and tended to have a greater incidence of being non-ambulatory at time of loading. Additionally, these pigs had a higher incidence of being non-ambulatory and injured at the packing plant. Pigs transported with 0.489 and 0.462 m²/pig floor space had less total transport losses compared to pigs that were transported with 0.396, 0.415, and 0.437 m²/pig. Similarly, Pilcher et al. (2011) examined transport losses (dead, non-ambulatory and noninjured, and non-ambulatory injured) and indicators of stress (open-mouth breathing, muscle tremors, and skin discoloration) of pigs subjected to different transportation periods (short = less than 1 h or long = 3 h) floor space allocation (0.396, 0.415, 0.437, 0.462, 0.489, or 0.520 m²/pig) during transport. There was a Floor space \times Journey time interaction for non-ambulatory and non-injured pigs and open-mouth breathing. Pigs allocated to 0.415 and 0.437 m²/pig floor space had greater incidence of being non-ambulatory and non-injured when experiencing the long journey time compared to the short journey time. The incidence of open-mouth breathing was greater in pigs allocated to 0.396, 0.415, and 0.437 m²/pig floor space when subjected to a short journey time compared to a long journey time. There were no main effects for Floor space or Journey time when examining transport losses or indicators of stress.

Another factor contributing to swine fatigue and transport losses is the environment during transportation. Fitzgerald et al. (2009) examined multiple transportation environments on transport

losses at Midwest commercial abattoirs. The authors concluded that there were more transport losses including dead, fatigued, and injured pigs during the hotter months of the year (June-July) compared to cooler months (November-December). Additionally, transport losses increased as the temperature-humidity index increased. Swine fatigue in relation to handling and transport suggests that pigs that are handled roughly or are required to move more during the transport process display more indicators of fatigue. Additionally, environmental factors including temperature and humidity can also play a role in non-ambulatory pigs during transport.

Ractopamine and muscle fatigue, stress, and transport losses in swine

There are a handful of studies that examine the effects of RAC on stress or fatigue in swine. Most of this literature used behavioral observations or blood metabolites to assess stress and fatigue. Peterson et al. (2001) fed pigs either 0, 5, or 7.5 mg/kg RAC and handled pigs gently, moderately, or aggressively in a simulated transportation model. The model consisted of moving pigs 50 m with an electric prod with 0, 4, or 8 shocks, transporting the pigs on a trailer for 1 h, and then moving the pigs 125 m with a paddle and sorting board. This study reported that pigs fed 7.5 mg/kg RAC had increased plasma epinephrine levels compared to the other RAC levels, indicating increased stress levels; however, plasma lactate, cortisol, and norepinephrine were not different between dietary treatments.

To examine behavior in gilts, Marchant-Forde et al. (2003) fed 72 gilts either 0 or 10 mg/kg RAC for 28 d. Gilt behavior was observed for inactivity, activity (walking, rooting, playing with pen mates or pen components), alertness, eating, standing, sitting, lying sternally, and lying laterally for one-22 h period every week until harvest (4 wk total). Additionally, during weekly weight measurements, the number of gilts exiting the pen voluntarily and the number of pushes required to move gilts were counted. The time to exit the pen, and time to enter the

weighing crate were also recorded. During weeks 1 and 2 of RAC administration, gilts fed 10 mg/kg RAC spent more time active, alert, and lying sternally than gilts fed 0 mg/kg RAC, and gilts fed 0 mg/kg spent more time lying laterally. Also within the first 2 weeks, the RAC gilts took longer to lie down than gilts not fed RAC. Over all weeks, gilts fed 10 mg/kg RAC spent more of their time active, eating, and lying sternally compared to gilts fed 0 mg/kg RAC. Across all wk, fewer gilts fed RAC exited pens voluntarily, required more pushes, and took longer time to exit pens and enter weighing crate compared to gilts not fed RAC.

Gillis et al. (2007) used 208 pigs in a simulated transportation procedure (consisting of a loading, transportation, unloading, lairage, and final drive process) to determine the effects of RAC in the diet. It was reported that after the final driving procedure, pigs fed 10 mg/kg RAC had increased serum lactate levels compared to pigs fed 0 mg/kg. Pigs fed 5 mg/kg RAC had increased levels of serum lactate compared to pig fed 0 mg/kg RAC, but decreased levels compared to pigs fed 10 mg/kg RAC. Within the same year, Swan et al. (2007) analyzed transport losses from pigs fed either 0, 5, or 10 mg/kg originating from Midwest or Southeast regions of the United States. The authors showed no difference in transport losses between the dietary treatments in the group coming from the Southeast; however, there were more transport losses in pigs fed 5 and 10 mg/kg RAC compared to pigs fed 0 mg/kg RAC in the group coming from the Midwest. The difference in transport losses could be due to the Midwest pigs experiencing longer transportation journeys by 61 minutes compared to Southeast pigs.

Rocha et al. (2013) videotaped the period of time used to move pigs from the pen to the transportation trailer. The authors then reviewed footage and recorded how many handler interactions (vocal sound, rattle noise, or physical including hands, battle, or boards) were required to walk pigs from their pens to the trailer for transportation. Additionally, behavioral

observations (slips, pig overlap, turns, backing up, vocalization, and refusal to walk) were also recorded by courting how many times each even occurred. Barrows were fed either 0 or 7.5 mg/kg RAC for 28 d. No differences were apparent for handler interactions between dietary treatment groups except for physical contact down the alley of the barn, where barrows fed 7.5 mg/kg required increased contact as encouragement. There was no difference in behavioral observations between treatment groups. Similarly, Athayde et al. (2013) examined the effects of feeding 0, 5, or 10 mg/kg RAC on behavioral and physiological characteristics of gilts and barrows. Thirteen behaviors (drinking, lying alone, lying in clusters, standing, nosing another pig, sitting, eating, biting another pig, walking, exploring, running, playing, and mounting other pen mates) were observed 3 d a week for 28 d on feed. Blood was collected at exsanguination for physiological measurements including lactate, cortisol, and creatine phosphokinase. Pigs fed 5 and 10 mg/kg RAC played more over the feeding period than pigs fed 0 mg/kg RAC. It was also reported that pigs fed 5 mg/kg RAC nosed other pigs more and spent more time drinking than pigs fed 0 mg/kg RAC. There were no differences between dietary treatment groups for lactate and cortisol levels; however, creatine phosphokinase levels were increased in pigs fed RAC. The authors suggest this increase could be due to increased muscle fiber diameter, or increased stress susceptibility, which extreme stress increases creatine phosphokinase in swine (Schmidt et al., 1974).

To determine effects of RAC on stress, James et al. (2013) subjected barrows and gilts to a stress model after being fed 0 or 20 mg/kg RAC for 28 d. The stress model included a gentle and aggressive handling treatments. The gentle course included moving pigs at a moderate pace with sorting boards 3 times through a 50 m course that included an alleyway and a 15° loading ramp. The aggressive course included moving pigs as fast as possible with an electric prod (1

shock per lap) 3 times through a 50 m course that included a narrowed alleyway and a 30° loading ramp. There were no RAC × Handling interactions for rectal temperature or any blood measure. Rectal temperature and blood were collected before and after each run. Rectal temperature tended to be increased after the test in pigs fed RAC. Blood cortisol and lactate dehydrogenase were increased after the study in pigs fed RAC compared to pigs not fed RAC. Additionally, pigs fed 20 mg/kg RAC had increased lactate both before and after the test compared to pigs fed 0 mg/kg RAC. Overall, pigs fed 20 mg/kg RAC were more susceptible to stress when handled roughly; however, one flaw to consider is this study included two different ramp inclines depending on whether gentle and aggressive handling treatments were used, which could be a confounding factor.

Recently, Puls et al. (2015) also used a simulated transport model to assess the effect of RAC on stress and fatigue in finishing pigs. One hundred and forty-four pigs were fed 0 or 10 mg/kg for 28 d and then subjected to a handling course consisting of 3 consecutive parts: 1) handling procedure 1 (aggressive), where pigs were individually moved 50 m with a sorting board and also received 8 shocks with an electric prod, 2) transportation procedure, where pigs were loaded together onto a trailer with paddles and sorting boards and then experienced a 30 min transportation ride, and 3) handling procedure 2 (gentle), where pigs were individually moved 100 m with a paddle and sorting board. Lactate values were increased in RAC pigs compared to pigs not fed RAC before and after the simulation. Epinephrine was also elevated in RAC pigs after the final handling procedure. Based on a subjective scale, physical indicators of stress (muscle tremors, open-mouth breathing, skin discoloration and vocalization) and transport losses were not different between dietary treatment groups; however, pigs fed RAC were harder to handle after the final handling procedure compared to pigs not fed RAC. Additionally, of the

pigs that did not finish the final handling procedure, pigs fed RAC walked a shorter distance compared pigs not fed RAC. Overall, only a few physiological changes were observed when pigs fed RAC were handled roughly, but the increased baseline lactate level in RAC before simulated transport poses concern that the study is valid when examining response to transportation.

In another study, where the same transportation simulation was used, Peterson et al. (2015) fed pigs 0, 5, or 7.5 mg/kg RAC. Based on a subjective scale, physical indicators of stress (muscle tremors, open-mouth breathing, skin discoloration and vocalization) were not different between dietary treatment groups; however, pigs fed 7.5 mg/kg RAC were had increased transport losses compared to pigs fed 0 and 5 mg/kg RAC. Epinephrine was elevated in pigs fed 7.5 mg/kg RAC compared to pigs fed 0 mg/kg RAC. Lactate concentration was not different between dietary treatment groups. This study suggests increased transport losses in pigs fed RAC; however, other indicators of stress including observational fatigue characteristics and lactate production were not observed to indicate the transport losses were due to pigs becoming fatigued.

Although data is variable when it comes to examining effects of RAC on muscle stress, muscle fatigue, and transport losses, the literature suggests RAC negatively affects swine transportation when pigs are handled aggressively.

Electromyography

Electromyography (**EMG**) measures the electrical signals of muscles. This system has many useful applications including measuring normal muscle function during specific movements and postures, muscle function activity during sports movements, occupational and rehabilitation movements, maternal muscle activity, and weight loading studies pertaining to back pain (Clarys et al., 2010). Additionally, EMG can also be useful in measuring fatigue

during physical activity. Electromyography measures the amplitude, or the electrical activity associated with recruitment of a muscle by either integrated EMG (**iEMG**) or root mean square (**RMS**). The amplitude includes muscle fiber recruitment rate and motor neuron firing rate (Enoka and Stuart, 1992), even though it is impossible to distinguish between the two with EMG analysis. The frequency characteristics of the EMG can be measured with either as mean power frequency (**MPF**) or median power frequency (**MdPF**). The frequency includes the action potential conduction velocity (Hagg, 1992). Electromyography is typically used in human kinesiology research; however, it has been used to measure muscle function in sheep (Girsch et al., 1995; Cockram et al., 2012), horses (Robert et al., 2003), goats (Lee et al., 2013), and cattle (Rajapaksha and Tucker, 2014).

Typically as a muscle fatigues, the frequency content of the muscle signal (as MDF or MdPF) decreases, meaning action potential conduction velocity slows (Hagg, 1992). Additionally, animals with more fast-twitch fibers have a greater rate of frequency decline, due to the decreased fatigue resistant properties of fast-twitch fibers (Hakkinen and Komi, 1986; Gerdle et al., 1991). In humans during repeated resistance training, Jenkins et al. (2015) reported decreased frequency in the leg muscles with increasing repetition. Cockram et al. (2012) reported that sheep displayed a reduction in MdPF in the *Semitendinosus* during prolonged slow treadmill walking. In human performance literature, amplitude values in the *Vastus lateralis* decrease during repeated sprints designed to induce fatigue (Mendez-Villanueva et al., 2008). Decreased muscle fiber activation (RMS values) during repeated physical activity in humans were also reported by Kinugasta et al. (2004) and Racinais et al. (2007); however, in contrast with these studies, there are also a handful of articles that report no difference in muscle fiber recruitment as motor neuron firing rate during repeated exercise (Hautier et al., 2000; Billaut et al., 2005).

The first study to use EMG in livestock was conducted by Girsch et al. (1995). These authors used an invasive form of EMG, where the *Latissimus dorsi* and Thoracodorsal nerve were exposed in 6 anesthetized sheep. Four stimulation electrodes were placed on the Thoracodorsal nerve and 3 sensor electrodes were placed on the *Latissimus* muscle. Stimulation was applied to the nerve with increasing force from 0.2 mA to 4 mA. The results revealed a strong correlation with force and amplitude of the muscle signal. It can be hypothesized that the signal increased consistently, but did not decline because fatigue was never reached.

Contrary to the previous study, Robert et al. (2000) measured iEMG in trotting horses by surface EMG, which is non-invasive protocol. The EMG of the *Gluteus medius* and *Tensor fasciae latae* muscles were measure during various speeds and inclines as horses trotted on a treadmill. Speeds varied from 3.5 to 6 m/s and the incline was set at 0, 3, or 6% grade. Horses warmed up walking for 10 min and were then subjected to a 10 min trot at the predetermined speed and incline. With increased speed and incline, the iEMG of both muscles were increased depicting an increase in motor neuron firing rate or number of motor units recruited, and/or motor neuron firing rate; however, these horses were all subjected to physical activity that lasted only 10 min where muscle fatigue was not reached. It is possible that if the horses ran to fatigue as opposed to a set time duration, the iEMG signal would decrease indicating fatigue.

While Girsch et al. (1995) and Robert et al. (2000) simply examined EMG in livestock during muscle stimulation, Cockram et al. (2012) tried to identify EMG in response to fatigue in sheep. Sheep *Semitendinosus* and *Tensor fasciae latae* EMG were measured while sheep walked on a treadmill for either 5 h or for two-10 min. The authors found no differences in MPF of the *Tensor fasciae latae* between groups, but there was a decrease in the MPF in the *Semitendinosus*

for sheep walked for 5 h compared to sheep walked two-10 min sessions. The decrease in frequency is indicative of muscle fatigue by action potential conduction velocity.

Lee et al. (2013) surgically embedded EMG transducers on the lateral and medial *Gastrocnemii* muscles of goats and then recorded the EMG readings during walking (1.1-1.4 m/s), trotting (1.7-2.8 m/s), and galloping (3.3-4.9 m/s). The results demonstrated that as the goats increased speed, faster motor units were recruited. In a different species, Rajapaksha and Tucker (2014) used EMG as a way to determine the short term effects of sloped floors on dairy cattle. Sixteen dairy cows were subjected to standing on floors with 0, 3, 6, and 9% slope for 90 min. Each cow was rotated between the 4 treatments, so that each cow experienced each slope once and tests were done approximately 2 d apart. Surface EMG sensors were placed on the middle *Gluteal* and *Biceps femoris* during each session. There were no differences in amplitude or frequency from the start to end of the 90 min session for either muscle between treatments; however, the frequency values were decreased in the *Biceps femoris* muscle compared to the *Gluteal* muscle regardless of slope. This suggests that the anatomy of different muscles can cause differences in action potential conduction velocity.

Problems with surface electromyography

Although EMG can be helpful when studying fatigue, there are a number of issues that can interfere with the electrical signal. One of the main issues when using EMG to observe muscle activity is muscle crosstalk. This consists of signals of neighboring muscles interfering with signals from the target muscle. The amount of crosstalk signal at the target muscle has been identified as high as 16% (de Luca and Merletti, 1988; Koh and Grabiner, 1992). Crosstalk can occur when the spacing between electrodes on a sensor are spaced too far apart or when the sensor is located incorrectly on the muscle. The electrodes on a surface sensor each pick up

signals separately, and then these signals are subtracted and amplified to create a single result.

The further apart the electrodes on the sensor are, the greater the cross talk is, since the cross talk increases as the ability to pick up signals farther away increases (Roeleveld et al., 1997). In order to decrease the instance of crosstalk from neighboring muscles, electrodes should be placed on the belly of the target muscle as opposed to the perimeter of the muscle. Placing the sensor in the middle of the muscle increases the chances that the signals are coming from the target muscle alone (de Luca et al., 2010).

The size of the electrodes on the surface sensor also play a role on EMG signal variation. The electrode to skin ratio should be minimized to create a greater and more exact signal from the target muscle (Godin et al., 1991). The skin where the sensor is placed should be well cleaned and dry before application (Clancy et al., 2002). Another factor contributing to EMG issues is the level of subcutaneous fat thickness and skinfold measurements between sensor and target muscle. Minetto et al. (2013) reported obesity negatively affected the EMG signal. Additionally, Pinvicero et al. (2001) hypothesized skinfold thickness also negatively affected EMG signal during data recording. It can be concluded more layers that exist between the EMG sensor and the muscle, the harder it is for the signal to be transmitted. In humans, Fuglevalend et al. (1992) reported that EMG technology transmits better signals in males than females because in general, females have more subcutaneous fat than males (Hattori et al., 1991).

Chapter 3 - Use of electromyography to detect muscle exhaustion in finishing barrows fed ractopamine-HCl

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Abstract

The objectives of this study were to determine the effects of dietary ractopamine-HCl (**RAC**) on muscle fiber characteristics and electromyography (**EMG**) measures of finishing barrow exhaustion when barrows were subjected to increased levels of activity. Barrows ($n = 34$; initial BW = 92 ± 2 kg) were assigned to one of two treatments: a commercial finishing diet containing 0 mg/kg (**CON**) or a diet formulated to meet the requirements of finishing barrows fed 10 mg/kg RAC (**RAC+**). After 32 d on feed, barrows were individually moved around a track at 0.79 m/s until subjectively exhausted. Wireless EMG sensors were affixed to the *Deltoideus* (**DT**), *Triceps brachii lateral head* (**TLH**), *Tensor fasciae latae* (**TFL**), and *Semitendinosus* (**ST**) muscles to measure median power frequency (**MdPF**) and root mean square (**RMS**) as indicators of action potential conduction velocity and muscle fiber recruitment, respectively. After harvest, samples of each muscle were collected for fiber type, succinate dehydrogenase (**SDH**), and capillary density analysis. Speed was not different ($P = 0.82$) between treatments, but RAC+ barrows reached subjective exhaustion earlier and covered less distance than CON barrows ($P < 0.01$). There were no Treatment \times Muscle interactions or

Treatment effects for end-point MdPF values ($P > 0.29$). There was a Treatment \times Muscle interaction ($P = 0.04$) for end-point RMS values. The RAC diet did not change end-point RMS values in the DT or TLH ($P > 0.37$); however, the diet tended to decrease and increase end-point RMS in the ST and TFL, respectively ($P < 0.07$). There were no Treatment \times Muscle interactions for fiber type, SDH, or capillary density measures ($P > 0.10$). Muscles of RAC+ barrows tended to have less type I fibers and more capillaries per fiber ($P < 0.07$). Type I and IIA fibers of RAC+ barrows were larger ($P < 0.07$). Compared to all other muscles, the ST had more ($P < 0.01$) type IIB fibers and larger type I, IIA, IIX fibers ($P < 0.01$). Type I, IIA, IIX fibers of the ST also contained less SDH compared to other muscles ($P < 0.01$). Barrows fed a RAC diet had increased time to subjective exhaustion due to loss of active muscle fibers in the ST, possibly due to fibers being larger and less oxidative in metabolism. Size increases in type I and IIA fibers with no change in oxidative capacity could also contribute to early exhaustion of RAC+ barrows. Overall, EMG technology can measure real-time muscle fiber loss to help explain subjective exhaustion in barrows.

Introduction

Fatigued pig syndrome has been used to loosely characterize pigs that suffer inordinately from transport stress in spite of having no obvious signs of disease or injury (Ritter et al., 2009). Typically, fatigued pigs may recover if rested for 2 to 3 h after transport (Ritter et al., 2006), but many pigs likely die before they can recover (Hamilton et al., 2004). Ritter et al. (2009) estimated fatigued pig syndrome may cause as much as \$46 million in losses annually. Although there is likely no single cause of this syndrome, use of feed additives designed to increase skeletal muscle growth during finishing may contribute.

Ractopamine-HCl (**RAC**), a β -adrenergic agonist fed during the finishing phase of swine production, improved ADG, feed efficiency, and lean muscle growth (Watkins et al., 1990; Moloney et al., 1991). Studies reported RAC increased the rate of fatigue when pigs were handled roughly; however, these studies used indirect measures of blood metabolites as indicators (James et al., 2013; Peterson et al., 2015; Puls et al. 2015). Dietary RAC shifted muscle fiber type from oxidative to glycolytic metabolism (Paulk et al., 2014), but the impact of fiber shifts on fatigue remain unexplored.

Wireless electromyography (**EMG**) has been used in human physiology to directly measure median power frequency (**MdPF**) and root mean square (**RMS**), which serve as indicators of action potential conduction velocity and muscle fiber recruitment, respectively (Broxterman et al., 2014). Fatigue in other livestock species has been measured via EMG (Girsch et al., 1995; Robert et al., 2000; Lee et al., 2013; Rajapaksha and Tucker, 2014), but these studies did not explore the biological characteristics of the muscle responsible for fatigue. Therefore, the objectives of this study were to use a RAC diet feeding model to induce muscle exhaustion in barrows, measure muscle fiber activity via EMG, and relate differences in exhaustion to muscle fiber type characteristic differences.

Materials and methods

The Kansas State University Institutional Animal Care and Use Committee approved the protocol used in this experiment.

Live animal management

Thirty-four finishing barrows (Pig Improvement Company 337 × 1050; Hendersonville, TN; initial BW 92 kg ± 2 kg) were housed in an environmentally controlled finishing barn at the Kansas State University Swine Teaching and Research Center (Manhattan, KS). Seven-days prior to beginning the feeding portion of the experiment, barrows were selected from the general population and moved into 7.4 m² individual pens with slatted-floors. Each pen was equipped with a wet/dry feeder that allowed *ad-libitum* access to feed and water. Twenty-four hours prior to treatment administration, barrows were weighed, stratified by weight, and allocated to one of two dietary treatments within each strata. Dietary treatments consisted of a conventional swine finishing diet containing 0 mg/kg RAC (**CON**) or a diet formulated to support a finishing barrow fed 10 mg/kg RAC (**RAC+**; Paylean, Elanco Animal Health, Greenfield, IN) according to NRC requirements (NRC, 2012). Diets were assayed to contain 8 to 9 mg/kg of RAC. Diets were fed during the final 35 d of feeding prior to harvest (Table 1). The experiment was repeated 3 times (2 periods with 10 barrows and 1 period with 14 barrows).

Performance test

On d 32 of feeding, barrows were randomly assigned a test order and individually subjected to a performance test. Before each test, the barrow was removed from its pen, restrained, and 15 mL of blood was collected from the jugular vein into 2 vacutainer tubes containing sodium heparin or sodium fluoride/potassium oxalate (BD Vacutainer, Franklin Lakes, NJ). Using hair clippers (305 Speed Feed Trim Kit; Laube Co., Inc., Oxnard, CA), the

hair covering the *Deltoideus* (**DT**), *Triceps brachii lateral head* (**TLH**), *Tensor fasciae latae* (**TFL**), and *Semitendinosus* (**ST**) muscles on the left side of the barrow was trimmed. The shaved areas were cleaned with 70% ethanol, air dried, and wireless EMG (Tringo EMG, Delsys, Natick, MA) electrodes were affixed to each muscle area so that the 4 contact bars arranged in a 2×2 orientation were placed perpendicular to the orientation of the muscle fibers. Data were sampled at 1,000 Hz/s and stored for later analysis.

Each barrow was walked at an average speed of 0.79 m/s around a circular track (perimeter = 30.67 m) by 3 handlers with sorting boards and paddles until subjective exhaustion was achieved. Subjective exhaustion was determined by the same handler who was blinded to treatment allocation for all 34 barrows. Subjective exhaustion was determined as the barrow stopping forward movement (not due to distraction or discomfort), which resulted in human application of pressure to the rump to resume movement, five times. If a barrow refused to continue forward movement after 20 s of encouragement during a single stop, exhaustion was also designated. Time and distance to exhaustion were recorded for each barrow and average speed was calculated based on laps/min. After each test, the barrow was restrained and 15 mL of blood was collected as described previously.

Plasma analysis

Within 10 min of each blood collection, tubes were centrifuged at $1,115 \times g$ for 10 min at 4°C. Plasma was transferred to 15 mL conical tubes, placed immediately on ice, and ultimately frozen at -80°C until analyzed. Plasma glucose and lactate were analyzed using a YSI 2300 STAT Plus Glucose and L-lactate Analyzer (YSI Inc., Yellow Springs, OH) with the use of a buffer containing disodium phosphate, monosodium phosphate, sodium benzoate, disodium EDTA, sodium chloride, and gentamicin sulfate (Part # 2537; YSI Inc.). Glucose analysis was

conducted using glucose membranes (Part# 2365; YSI Inc.) and 2 g/L glucose standard (Part# 2355; YSI Inc.), while lactate analysis used L-lactate membranes (Part# 2329; YSI, Inc.) and 30 mmol/L L-lactate standard (Part# 1530; YSI Inc.). Glutamate and glutamine were analyzed using an YSI 2700 SELECT Analyzer (YSI Inc.) and the same buffer described above. Glutamate membranes (Part# 2754; YSI Inc.), 5 mmol/L glutamate standard (Part# 2755; YSI Inc.), glutamine membranes (Part# 2735; YSI Inc.), and 5 mmol/L glutamine standard (Part# 2736; YSI Inc.) were used in the analysis.

Electromyography analysis

The methods of Broxterman et al. (2014) were followed with modifications for EMG analysis. Using a custom computer program in MATLAB Student R2011a (MathWorks, Inc., Natick, MA), raw EMG data were processed for each electrical burst corresponding with a muscle contraction by using a band-pass filter (13-400 Hz). The EMG frequency and amplitude characteristics were derived as MdPF and RMS, respectively. Data were averaged every 5 s during the performance test of each barrow. The MdPF and RMS values of each barrow were normalized to the average of all values of the individual barrow's own performance test, which is commonly done in human performance literature (Murray et al., 1985; Hunter et al., 2014). This average was assigned the value of 100% and denoted as the normalization value. Subsequent 5 s increments were divided by the normalization value and multiplied by 100 to yield a percentage of the MdPF or RMS normalization value. The percentages of the last 15 s before subjective exhaustion was reached by the barrow were averaged and utilized for statistical analysis as end-point MdPF and RMS values.

Harvest, sample collection, immunohistochemistry, and histology

After a 3 d rest period (35 d of feeding), barrows were transported to the Kansas State University Meats Laboratory (Manhattan, KS) for harvest under Federal inspection. After chilling for 24-h postmortem, carcass measurements were taken by trained university personnel. Marbling and color attributes were evaluated according to the National Pork Producers Council (NPPC) “Pork Quality Standards” (NPPC, 1999). One, 2.54 cm cores of the DT, TLH, TFL, and ST were taken from the left side of each carcass at the approximate EMG locations for muscle fiber type, succinate dehydrogenase (**SDH**), and capillary density analysis.

A 1-cm² portion of each core was embedded in tissue embedding media (Fisher Scientific, Pittsburgh, PA), cooled with super-cooled isopentane, and stored at -80°C until analysis. Two cryosections per slide, 0.5 mm apart, for each muscle sample were collected on 3 slides for fiber type (5-μm thick), capillary density analysis (5-μm thick), and SDH analysis (20-μm thick). All cryosections were collected on positively charged slides (Diamond White Glass; Globe Scientific Inc., Paramus, NJ).

The methods of Paulk et al. (2014) were followed for fiber type immunohistochemistry with modifications. Cryosections were incubated in 5% horse serum and 0.2% TritonX-100 in phosphate buffered saline (**PBS**) for 30 min to block all non-specific binding sites. Cryosections were incubated for 16 h at 4°C with a primary antibody solution consisting of blocking solution and 1:500 α-dystrophin (Thermo Scientific, Waltham, MA), 1:10 supernatant myosin heavy chain, slow, IgG2b (BA-D5; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA), 1:10 supernatant myosin heavy chain, type IIA, IgG1 (SC-71; Developmental Studies Hybridoma Bank), and 1:10 supernatant myosin heavy chain, type IIB, IgM (BF-F3; Developmental Studies Hybridoma Bank). Following incubation, cryosections were washed 3 times for 5 min with PBS and incubated for 30 min with secondary antibodies in blocking

solution containing: 1:1000 Alexa-Flour 488 Goat anti-mouse IgM (Life Technologies, Carlsbad, CA) for BF-F3, 1:1,000 Alexa-Flour 594 goat anti-mouse IgG1 (Life Technologies) for SC-71, 1:1,000 Alexa-Flour 633 goat anti-mouse IgG_{2b} (Life Technologies) for BAD-5, 1:1,000 Alexa-Flour 594 goat-anti rabbit heavy and light chains (Life Technologies) for α -dystrophin, 1:1,000 Alexa-Flour 488 goat-anti rabbit heavy and light chains (Life Technologies) for α -dystrophin, and 1:1,000 Hoechst Dye 33342 (Life Technologies). After washing in PBS 3 times for 5 min, 5 μ l of 9:1 glycerol in PBS was placed on each cryosection and they were then cover-slipped for imaging.

Slides with cryosections for SDH staining were incubated at 37°C for 1 h in a pre-warmed incubation solution containing 50% nitro-blue tetrazolium solution (1% nitro-blue tetrazolium in Milli-Q [**MQ**] water), 25% phosphate buffer (20% potassium phosphate monohydrate, 11% disodium hydrogen phosphate in MQ water), and 25% sodium succinate solution (2.7% sodium succinate dibasic hexahydrate in MQ water). After washing in MQ water 3 times for 1 min each, 5 μ l of 9:1 glycerol in PBS was placed on each cryosection and they were cover-slipped for imaging.

Cryosections for capillary density were blocked as described above. Cryosections were then incubated for 1 h in a primary antibody solution containing blocking solution and 1:50 PECAM-1 mouse monoclonal IgG2a (Santa Cruz Biotechnology Inc., Dallas, TX) and 1:500 α -dystrophin (Thermo Scientific). Cryosections were washed 3 times for 5 min with PBS and incubated for 30 min with secondary antibodies in blocking solution containing 1:1,000 Alexa-Flour 594 goat-anti mouse IgG heavy and light chains (Life Technologies) for Platelet Endothelial Cell Adhesion Molecule-1 (**PECAM-1**) and 1:1,000 Alexa-Flour 488 goat-anti

rabbit heavy and light chains (Life Technologies) for α -dystrophin. After washing in PBS 3 times for 5 min, cryosections were covered with 5 μ l of 9:1 glycerol, and cover-slipped for imaging.

All cryosections were imaged at 10 \times magnification with a Nikon Elipse TI-U inverted microscope (Nikon Instruments Inc., Melville, NY). Muscle fiber type and capillary photomicrographs were taken with a Nikon DS-QiMC digital camera (Nikon Instruments Inc.) and SDH photomicrographs were captured with Nikon DS-Fi1 color digital camera (Nikon Instruments Inc.). White light intensity was kept constant for SDH photomicrographs. All analysis was conducted using NIS-Elements Imaging software (Basic Research, 3.3; Nikon Instruments Inc.). An average of 500 muscle fibers were analyzed for myosin heavy chain isoform distribution and fiber cross-sectional area (**CSA**). Fibers that stained exclusively positive for BAD-5, SC-71, and BF-F3 were labeled type I, type IIA, and IIB, respectively. Fibers that stained positive for both SC-71 and B-FF3 were labeled as type IIX fibers (Fig. 1; Paulk et al. 2014). Cross-sectional area of muscle fibers was determined as the area within the dystrophin border. Capillary density was determined by dividing the total number of capillaries by the total number of muscle fibers within a photomicrograph to yield a ratio. A minimum of 500 fibers and their associated capillaries were counted with all fibers counted in a photomicrograph. To determine SDH intensity, a minimum of 200 fibers were identified by type through immunohistochemistry, and the mean intensity of the SDH stain was determined by the software. A value of 0 indicated black (most intense) staining, while a value of 250 indicated white (least intense) staining.

Statistics

All analyses were performed utilizing Animal as the experimental unit and Experimental Period as Block. Finishing performance, carcass, and performance test data were analyzed as a

randomized complete block design. Treatment served as the fixed effect and Block as the random effects. Blood measures were analyzed as a randomized complete block design with repeated measures. Fixed and random effects were the same as described above and Time served as the repeated measure, with Animal as the subject, and compound symmetry as the covariance structure. Muscle immunohistochemistry and histochemistry, and EMG data were analyzed as a split-plot design. The whole-plot consisted of Animal and Muscle within Animal served as the sub-plot. Fixed effects included Treatment, Muscle, and their interaction, and random effects were Animal \times Treatment and Block. All models were analyzed using the MIXED procedure of SAS 9.3 (SAS Institute Inc., Cary, NC). Pairwise comparisons between the least square means of the factor level comparisons were computed using the PDIF option of the LSMEANS statement. Statistical significance was determined at $P \leq 0.05$ and tendencies were determined at $0.05 > P \geq 0.10$.

Results

Growth performance and carcass characteristics

Finishing performance and carcass measurements are presented in Table 2. Barrows' initial BW, final BW, and ADG were not different between treatment groups ($P > 0.15$). Barrows from the RAC+ treatment had reduced ($P = 0.03$) ADFI compared to CON barrows, which resulted in RAC+ barrows possessing greater ($P < 0.01$) G:F. Dietary treatment did not affect HCW, dressing percent, and all s.c. fat measurements ($P > 0.11$). Loin eye area (**LEA**) and color were not affected by dietary treatment ($P > 0.14$); however, there was less ($P < 0.01$) marbling in the loins of RAC+ barrows compared to loins from CON barrows.

Performance test and electromyography analysis

There was no difference ($P = 0.82$) in the speed at which barrows moved around the track; however, barrows from the RAC+ treatment reached subjective exhaustion earlier and covered less distance than CON barrows ($P < 0.01$; Table 3). There were no Treatment \times Muscle interaction, Treatment, or Muscle effects for end-point MdPF values ($P > 0.42$; Fig. 2a). There was a Treatment \times Muscle interaction ($P = 0.04$) for end-point RMS values (Fig. 2b). Dietary treatment did not affect end-point RMS values of the DT or TLH ($P > 0.37$), but RAC+ barrows tended to have decreased ($P = 0.06$) end-point RMS values in the ST and tended to have increased ($P = 0.07$) end-point RMS values in the TFL compared to CON barrows. There were no Treatment effects or Muscle effects for end-point RMS values ($P > 0.15$).

Plasma analysis

There were no Treatment \times Time interactions for plasma lactate, glucose, glutamate, and glutamine values ($P > 0.16$; Table 3). There were no Treatment effects on circulating lactate, glutamine, or glutamate ($P > 0.39$); however, there was a tendency for the CON barrows to have elevated ($P = 0.09$) glucose compared to RAC+ barrows. There were Time effects on all plasma measurements with greater lactate, glucose, and glutamate circulating after the performance test for all barrows ($P < 0.01$), while glutamine was reduced ($P < 0.01$).

Immunohistochemistry and histology

Treatment, Muscle, and their interaction effects on immunohistological and histological measurements were analyzed within each muscle fiber isoform, except for capillary density which was analyzed over all fibers (Table 4). There were no Treatment \times Muscle interactions for the percentage of each fiber type ($P > 0.12$), except a tendency ($P = 0.10$) for type IIA percentage. Within DT, TLH, and ST muscles, type IIA fiber percentage was not affected by

dietary treatment ($P > 0.39$); however within the TFL, RAC+ barrows had greater ($P = 0.02$) percentage of type IIA fibers than CON barrows. Over all muscles, Treatment did not affect the percentage of type IIA, IIX, and IIB fibers ($P > 0.13$), but CON barrows tended to have more ($P = 0.07$) type I fibers than RAC+ barrows. There were Muscle effects on type I and IIB muscle fibers ($P < 0.01$). The DT had more type I fibers than all other muscles ($P < 0.04$), the TLH had more type I fibers than the ST and TFL ($P < 0.01$), and there was a tendency for the TFL to have more ($P = 0.09$) type I fibers than the ST. Type IIA and IIX fiber percentage tended to be affected by Muscle ($P < 0.10$). The DT and TLH had greater percentage of type IIA fibers than the ST ($P < 0.03$), while there were no differences between the other muscles ($P > 0.15$). There were more ($P < 0.01$) type IIX fibers in the DT compared to the ST, the DT tended to have more ($P = 0.10$) type IIX fibers than the TFL, and there were no differences between the other muscles ($P > 0.17$). There were more type IIB fibers in the ST compared to the other muscles ($P < 0.01$), the TFL contained more type IIB fibers than the DT and TLH ($P < 0.01$), and the TLH had more ($P = 0.01$) type IIB fibers than the DT.

There were no Treatment \times Muscle interactions for the CSA of any fiber type ($P > 0.36$). Over all muscles, Treatment did not affect the CSA of type IIX and IIB fibers ($P > 0.11$), but RAC+ barrows tended to have larger ($P = 0.07$) type I fibers and larger ($P = 0.03$) type IIA fibers compared to CON barrows. There were Muscle effects on the CSA of type I, IIA, and IIX fibers ($P < 0.01$), but no effect ($P = 0.67$) on type IIB fibers. Within type I fibers, the ST had larger fibers than all other muscles ($P < 0.01$), the DT and TLH had larger fibers than the TFL ($P < 0.01$), and there was no size difference between the DT and TLH ($P = 0.70$). There were larger type IIA fibers in the ST compared to other muscles ($P < 0.01$), the TLH tended to have larger type IIA fibers than the TFL ($P = 0.08$), and there were no differences between other muscles (P

> 0.33). Within type IIX fibers, the ST had larger fibers compared to all other muscles ($P < 0.01$) and there were no differences between the other muscles ($P > 0.13$).

There were no Treatment \times Muscle interactions for the SDH intensity for all fiber types ($P > 0.21$). Across all muscles, there were no Treatment effects on SDH intensity ($P > 0.21$). There were Muscle effects for SDH intensity within type I, IIA, and IIX fibers ($P < 0.01$), with no effect for type IIB fibers ($P = 0.35$). The ST had less intense SDH staining of the type I fibers compared to all other muscles ($P < 0.01$), there was a tendency for the TFL to have less ($P = 0.10$) SDH staining intensity than the DT for type I fibers, and there were no differences between the other muscles ($P > 0.15$). Within type IIA and IIX fibers, the ST had least intense SDH staining than the other muscles ($P < 0.01$), but there were no differences between the other muscles ($P > 0.41$). There were no Treatment \times Muscle interactions or Muscle effects for capillary density ($P > 0.17$); however, the RAC+ barrows had more ($P = 0.04$) capillaries per fiber compared CON barrows.

Discussion

Growth performance and carcass characteristics

Swine producers commonly feed RAC during the final days of finishing because of the positive effects the compound elicits on finishing pig performance and carcass characteristics. In the current study, a RAC diet fed during the final 35 d of feeding did not affect ADG, but did decrease ADFI by 9% and increased G:F by 16%. While the current study fed barrows individually, the majority of RAC studies fed groups of pigs and differences in study design could be the reason for variable responses between studies (Armstrong et al., 2004; Carr et al., 2005; Paulk et al., 2014). Carr et al. (2005) reported that RAC fed barrows had increases of 16% in ADG and 19% in G:F, but no differences in ADFI. Utilizing barrows and gilts raised at the

same farm as the current study, Paulk et al. (2015) reported RAC fed during the final 35 d of feeding increased ADG by 11%, reduced ADFI by 6%, and increased G:F by 17%. In contrast to these studies, Armstrong et al. (2004) reported that RAC fed to barrows did not influence ADG or ADFI, while Stites et al. (1991) showed that regardless of RAC level in the feed, ADFI was not affected. Overall, significant finishing performance results of the current study are similar to published literature.

Because RAC is a repartitioning agent that directs nutrients away from adipose growth and toward muscle growth, carcasses commonly exhibit more muscle and less fat. Crome et al. (1996) demonstrated that increasing the concentration of RAC in the diet resulted in a linear LEA increase and a linear decrease in 1st rib, 10th rib, and last lumbar backfat thicknesses. Paulk et al. (2015) reported increases in HCW of 3%, 6% increase of loin depth, and a corresponding 16% decrease in backfat depth when pigs were exposed to the same RAC feeding regimen as the current study. Burnett et al. (2016) showed that 10 mg/kg RAC tended to increase HCW by 3%, increased LEA by 10%, but only tended to decrease 10th and last rib backfat. In the current study all carcass measures were unaffected by a RAC diet except marbling score was reduced 27% in RAC+ barrows. In agreement, Carr et al. (2005) found RAC did not affect all s.c. backfat measures and Main et al. (2009) reported 10 mg/kg RAC did not affect loin depth. Apple et al. (2008) reported that RAC increased marbling, while other studies demonstrate RAC does not affect marbling (Crome et al., 1996; Carr et al., 2005, Bohrer, et al., 2013); however, Armstrong et al. (2004) found that RAC decreased the amount of marbling. Therefore, these data would indicate the adipose response to a RAC diet is variable between depots. Overall, the lack of significant dietary RAC carcass responses in the current study differs from the majority of the

literature. These results may be due to the reduced animal numbers employed in the study, as carcass effects were not what the study was designed to detect.

Performance test

Previously, pigs fed RAC are categorized as being difficult to handle. Rocha et al. (2013) reported RAC barrows required more physical contact in order to move during handling and transportation. Additionally, Marchant-Forde et al. (2003) found pigs fed RAC took longer to voluntarily exit pens during weekly weight measurements, thus making them more difficult to handle. A handful of studies exist that examined the effects of rough handling and dietary RAC on exhaustion. James et al. (2013) concluded pigs fed 20 mg/kg RAC (2-fold current label directions) are more prone to stress, as indicated by elevated rectal temperature, decreased blood pH, and increased lactate dehydrogenase concentration, when handled aggressively. Recently, based on a subjective handling scale, Puls et al. (2015) reported pigs fed RAC tended to be harder to handle and of the pigs that did not finish the last 100 m of a transportation simulation course, pigs fed RAC walked 7% shorter distance compared to pigs not fed RAC. Conversely, in another study where the same transportation simulation model was employed, Peterson et al. (2015) found that pigs subjected to different levels of RAC and handling intensity did not display diverse fatigability characteristics, such as open-mouth breathing, skin discoloration, muscle tremors, and vocalization. In the current study, RAC+ barrows undertook ambulatory movement for almost 2 min less and 200 m shorter distance compared to CON barrows. Importantly, these findings were not due to the speed at which the pigs walked; therefore, differences may be due muscle physiological changes induced by the RAC diet.

Plasma glucose, lactate, glutamate, and glutamine

Blood parameters have been utilized as indirect measures of muscle fatigue in human and livestock physical activity research. In humans, plasma glucose levels elevate with exercise due to increased hepatic glucose production to meet the amplified demand of glucose uptake by active muscle fibers (Kj  er, 1988). Plasma lactate concentrations are elevated with increasing levels of exercise after the lactate threshold is reached (Svedahl and MacIntosh, 2003) and will increase precipitously until exhaustion if exercise is sufficiently intense (Poole et al., 1988). Increased glutamate and decreased plasma glutamine after prolonged exercise in humans are often indicators of muscle damage (Keast et al., 1995; Walsh et al., 1998; Leibowitz et al., 2012). Each blood plasma parameter in the current study changed after performance testing where circulating glucose (23%), lactate (139%), and glutamate (51%) were elevated and glutamine (11%) was depressed. This would indicate that glucose levels were increased to meet the demand for movement, the performance test stimulated elevated lactate production, most likely from muscles, and muscles were becoming damaged as indicated by glutamate and glutamine trends. When pigs are subjected to simulated transportation courses, blood glucose and lactate levels rise (James et al., 2013; Peterson et al., 2015; Puls et al., 2015). Additionally, Weiss et al. (1974) found that after 5 min of increased physical activity, both plasma glucose and plasma lactate were increased. Similar to the current study, Hackl et al. (2009) reported a 13% decrease in glutamine and a 68% increase in glutamate after an intense bout of exercise in trotting horses. The present circulating plasma metabolite data suggest that all barrows reached a similar state of exhaustion congruent with intense activity and overall accumulation of these metabolites were not affected by dietary RAC. These findings would indicate that indirect measures of exhaustion through blood metabolite measurement are not useful when subjecting animals to protocols not based on common time or distance traveled, but stimulating extreme levels of fatigue

Electromyography

To date no studies exist that utilize the current exhaustion model and EMG technology in pigs fed RAC diets. Electromyography sensors were attached to 4 muscles important to ambulatory movement in pigs; the DT and TLH of the forelimb and the TFL and ST of the hindlimb. The current study did not examine EMG measures across the duration of the performance test, but analyzed the last 15 s of movement for each barrow before subjective exhaustion occurred in order to identify the specific physiological mechanisms responsible for exhaustion. In the cascade of events that are responsible for muscle contraction, MdPF measures action potential conduction velocity or the velocity at which the motor unit action potential travels along muscle fibers during contraction (Soares et al., 2015). Typically as a muscle fatigues, MdPF decreases (Hagg, 1992). In humans during repeated resistance training, Jenkins et al. (2015) reported decreased frequency in leg muscles with increasing repetition. Cockram et al. (2012) reported sheep displayed a reduction in MdPF in the ST during prolonged slow treadmill walking, but the TFL was unaffected. In the current study, end-point values were not different between muscles or treatment. These findings indicate this segment of the muscle contraction process was not responsible for the onset of exhaustion.

Electromyography measurement of RMS serves as an indicator of muscle fiber recruitment (number of active muscle fibers) or motor neuron firing rate. While MdPF was unaffected by treatment, end-point RMS values indicate why RAC+ barrows became exhausted faster than CON barrows. There was a tendency for RAC+ barrows to have reduced end-point RMS values in the ST; thus indicating an increased loss in muscle fibers. Possibly in an effort to compensate for the loss of ST fibers, end-point RMS values in the TFL muscle of RAC+ barrows tended to increase, or the TFL recruited more fibers. In human performance literature, RMS values in the *Vastus lateralis* decreased during repeated sprints designed to induce fatigue

(Mendez-Villanueva et al., 2008). Decreased muscle fiber activation, indicated by RMS values, during repeated physical activity in humans were also reported by Kinugasa et al. (2004) and Racinais et al. (2007). Therefore, of the muscles analyzed, it is possible the reduction of ambulatory movement in pigs fed a RAC diet may be due to a reduction in muscle fiber recruitment in the ST, a muscle that is important in extending the hip, stifle and hock, and flexes the stifle when the leg is lifted (Jones et al., 2005).

Fiber type, succinate dehydrogenase, and capillary density

Over all muscles and barrows, there were 13% type I, 24% type IIA, 19% type IIX, and 44% type IIB fibers. This distribution is slightly different than those reported by Lefaucheur et al. (2002) and Paulk et al. (2014) who reported distributions of 8% type I, 11-14% type IIA, 25-32% type IIX, and 46-50% type IIB fibers in swine LM muscles. The small difference between studies could be due to the different muscles analyzed. The previous studies analyzed a support muscle while the current study examined main locomotive muscles. The histochemical and immunohistochemical analysis in this study would indicate there are differences in metabolism between the ST and the other muscles. The ST had the least type I and most type IIB fibers, the latter of which are highly glycolytic and are most susceptible to fatigue (Pette and Staron, 2001). Succinate dehydrogenase is found in the inner membrane of the mitochondria, where the ability to produce ATP is dependent on oxidative phosphorylation (Kern et al., 1999); therefore, a cell that is more oxidative in nature possesses more SDH. The ST had less SDH in type I, IIA, and IIX fibers compared to all other muscles indicating a reduced oxidative capacity. Finally, the ST contained larger type I, IIA, and IIX fibers than the other muscles. Muscles with larger fibers possess a reduced VO_2 max, the maximum oxygen consumption of the fiber, and muscles with

reduced VO₂ max are more easily fatigable (van Wessel et al., 2010). These findings provide further evidence to the importance of the ST in contributing to onset of exhaustion.

The fiber type and CSA response in LM of pigs supplemented RAC is well documented. Sainz et al. (1993) reported no difference in fiber percentage, but increases in CSA of type II fibers when pigs were fed 20 mg/kg RAC for various time periods. Depreux et al. (2002) showed no difference in type I, decreased type IIA and IIX, and increased type IIB fiber percentages in pigs fed 60 mg/kg RAC for 42 d. When pigs were fed 10 mg/kg RAC for 35 d, the percentage of type IIB fibers increased at the expense of type IIX fibers (Paulk et al., 2014) and Burnett et al. (2016) reported increased CSA of type IIA and IIX fibers. Aalhus et al. (1992) found fiber type and fiber size were affected consistently by dietary RAC across multiple muscles. Over all muscles in the current study, RAC+ barrows tended to have 15% less type I fibers and on average 15% larger type I and IIA fibers. These changes could possibly explain the increased rate at which RAC+ barrows became exhausted. As stated above, shifting fibers within muscles away from oxidative metabolism and increasing size, which could contribute to a reduced VO₂ max, should make muscles more easily fatigable.

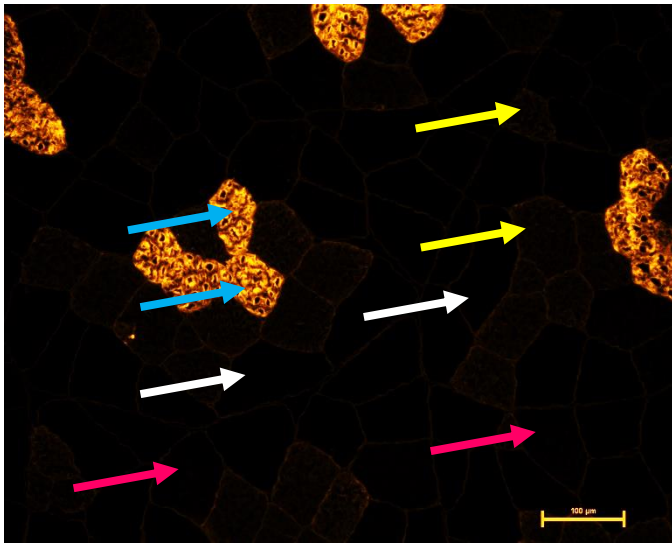
Capillaries allow for blood flow to and from the muscle, which is important for exchange of nutrients and waste (Aberle et al., 2003). Petersen et al. (1998) and Velotto et al. (2010) both reported capillary to fiber ratios that ranged from 0.90-1.50 for the *Psoas major*, *Rhomboideus*, and *Longissimus dorsi* muscles. The current study found ratios that ranged from 1.44-2.21 for all locomotor muscles examined. The RAC+ barrows had increased capillary density compared to CON barrows. There is evidence that β - agonists increase blood flow to muscles which results in hypertrophy (for review see Mersmann, 1998). Increased capillary density could be a mechanism by which the body is compensating for the increased size of

RAC+ barrow muscle fibers. Typically, there is increased oxygen delivery to oxidative fibers, which contain more capillaries than glycolytic fibers (Andersen, 1975; Hudlicka, 1985), meaning that muscles with highly fatigue resistant properties have increased capillary density. Therefore, the result of RAC+ barrows reaching exhaustion quicker than CON barrows is contrary to what would be expected for a muscle that has elevated capillary density. This could be due to mitochondria of the muscle cells in RAC+ barrows not being equipped to utilize the amount of oxygen delivered to muscle cells as indicated by SDH staining. The current study is the first of its kind to examine the effects of a β -agonist on the SDH intensity within muscle cells of livestock. Lynch et al. (1996) and Suzuki et al. (1997) reported that mice and rats fed a β -adrenergic agonist displayed decreased SDH concentrations in various muscles. Muscle cells become more fatigue resistant with increased SDH concentrations (Kugelberg and Lindegren, 1979; Martin and Edgerton, 1991). Dietary RAC did not affect the amount of SDH present in all fiber types despite changes in type I percentage and the size of type I and IIA fibers. The lack of a change in the oxidative capacity in the type I and IIA fibers, in the presence of increased CSA, may contribute to early onset of exhaustion in RAC+ barrows.

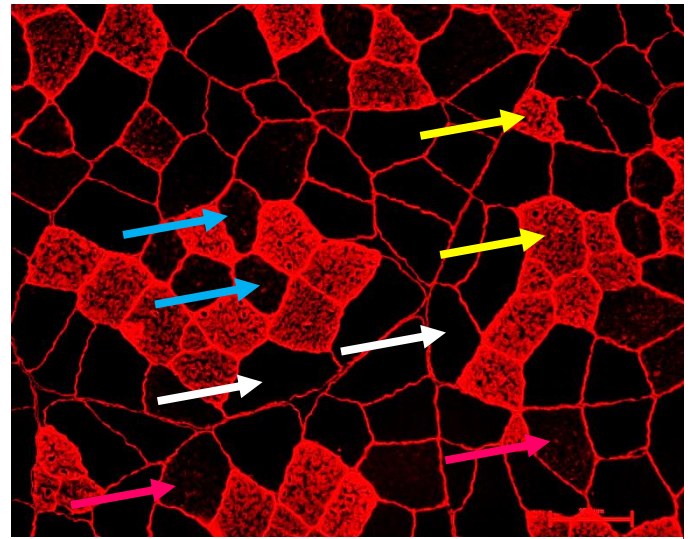
Conclusion

Feeding barrows a formulated diet containing 10 mg/kg RAC for 32 d decreased time and distance walked until exhaustion during a performance test. Electromyography analysis indicated that this occurred due to a loss of active muscle fibers in the ST, while the TFL attempted to compensate for these losses by increasing fiber recruitment. The ST possesses the physiology to become exhausted earlier since it contains larger fibers that are less oxidative in metabolism. While dietary inclusion of RAC increased the density of capillaries present in all muscles, SDH presence was not altered as type I and IIA fibers increased in CSA. Thus, the increases in the

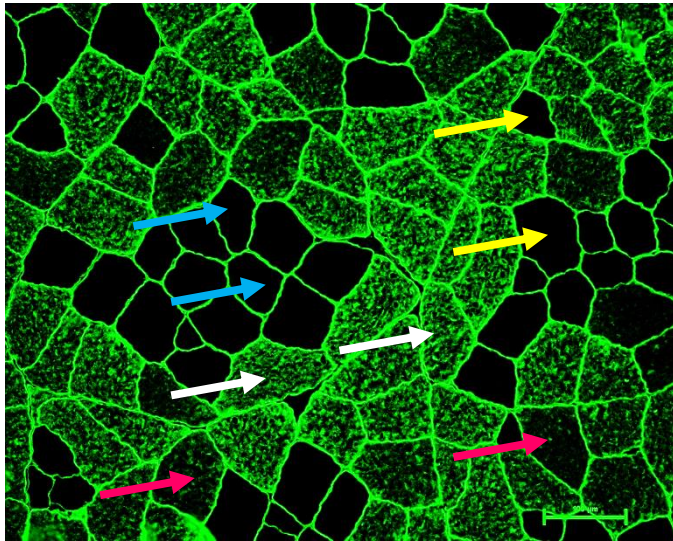
CSA of these fibers with no change in oxidative capacity may contribute to early onset exhaustion of barrows fed a RAC diet. Overall, EMG technology can be a valuable tool in directly measuring the muscle fiber recruitment patterns responsible for onset of muscle exhaustion in pigs.



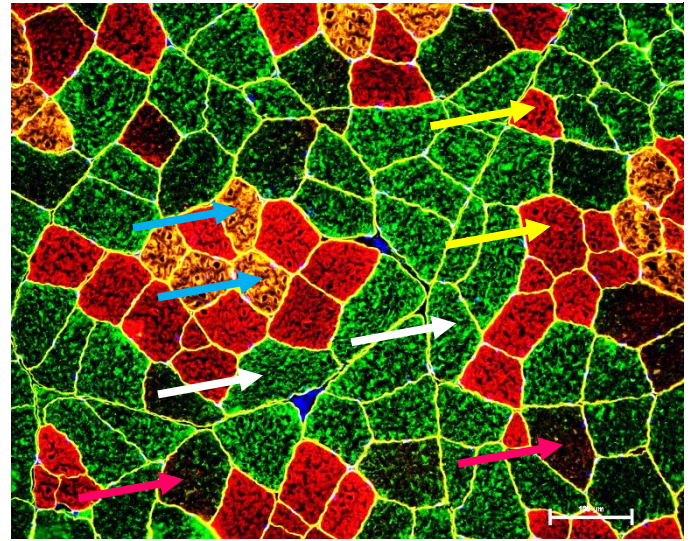
BA-D5 (Type I)



SC-71 (Type IIA)



BF-F3 (Type IIB)



Merged image

Figure 3.1 Representative photomicrographs of immunohistological fiber type staining pattern of porcine *Triceps brachii lateral head* (TLH) muscle. Fibers that stained positive for the BA-D5 (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) antibody were categorized as type I fibers (Blue arrows), fibers that stained positive for the SC-71 (Developmental Studies Hybridoma Bank) antibody only were categorized as type IIA fibers (Yellow arrows), fibers that stained positive for the BF-F3 (Developmental Studies Hybridoma Bank) antibody only were categorized as type IIB fibers (White arrows), and fibers that stained positive for both SC-71 and BF-F3 were categorized as type IIX fibers (Pink arrows; Paulk et al., 2014). Scale bars = 100 μ m.

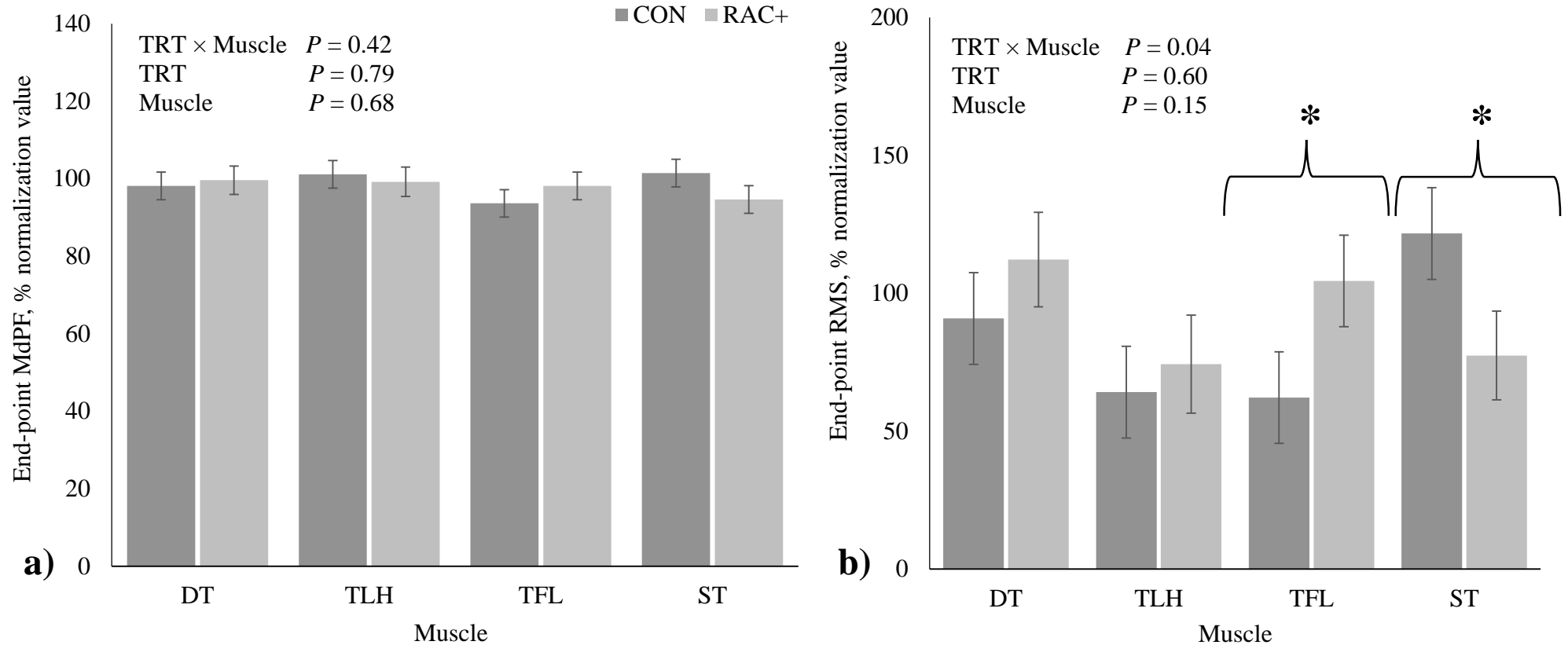


Figure 3.2 Electromyography (EMG) end-point a) median power frequency (MdPF) and b) root mean square (RMS) values of four muscles of pigs subjected to a performance test following 32 d of feeding a conventional swine finishing diet containing 0 mg/kg ractopamine-HCl (CON; Paylean; Elanco Animal Health, Greenfield, IN) or a diet formulated to support a finishing barrow fed 10 mg/kg ractopamine-HCl (RAC+). Barrows were removed from pens and EMG sensors were attached to the *Deltoideus* (DT), *Triceps brachii lateral head* (TLH), *Tensor fasciae latae* (TFL), and the *Semitendinosus* (ST) muscles. Barrows were individually walked around a 30 m track at 0.79 m/s until subjective exhaustion occurred. Exhaustion was determined as the barrow stopping forward movement (not due to distraction or fear), which resulted in human application of pressure to the rump to resume movement, five times. If a barrow refused to continue after 20 s of encouragement, exhaustion was also designated. Data were averaged every 5 s during the performance test of each barrow. The MdPF and RMS values of each barrow were normalized to the average of all values of the individual barrow's own performance test, which is commonly done human performance literature (Murray et al., 1985; Hunter et al., 2014). This average was assigned the value of 100% and denoted as the normalization value. Subsequent 5 s increments were divided by the normalization value and multiplied by 100 to yield a percentage of the MdPF or RMS normalization value. The percentages of the last 15 s before subjective exhaustion was reached by the barrow were averaged and utilized for statistical analysis as end-point MdPF and RMS values.*denotes mean tend to differ within muscle ($P < 0.07$).

Table 3.1 Diet composition (as-fed basis)¹

Item	Control	RAC ²
Ingredient, %		
Corn	82.93	75.17
Soybean meal	14.93	22.40
Calcium carbonate	1.00	0.98
Monocalcium phosphate	0.30	0.25
Salt	0.35	0.35
L-Lysine HCl	0.22	0.28
DL-Methionine	----	0.06
L-Threonine	0.05	0.10
Ractopamine-HCl 4.95 g/kg	----	0.20
Trace mineral premix ³	0.10	0.10
Vitamin premix ⁴	0.10	0.10
Phytase ⁵	0.01	0.01
Total	100	100
Calculated analysis, %		
Standardized ileal digestible (SID) amino acids, %		
Lys	0.72	0.95
Ile:Lys	66	63
Leu:Lys	162	141
Met:Lys	30	33
Met and Cys:Lys	59	59
Thr:Lys	65	65
Trp:lys	18.0	18.0
Val:Lys	76	70
Total lysine, %	0.82	1.07
ME, Mcal/kg	3.32	3.31
SID Lysine: ME, g/Mcal	2.17	2.87
CP, %	14.2	17.3
Ca, %	0.52	0.52
P, %	0.39	0.41
Available P, %	0.22	0.22

¹Diets were fed in meal form for the duration of the experiment.

²RAC = ractopamine-HCl. (Paylean; Elanco Animal Health, Geenfield, IN) fed during the final 35 d of feeding.

³Trace mineral premix provided 16.53 mg Mn, 55.06 mg Fe, 55.06 mg Zn, 8.25 mg CU, 0.15 mg I, and 0.15 mg Se per kilogram of the complete diet.

⁴Vitamin premix provided 3,307 IU vitamin A, 413 IU vitamin D₃, 13 IU vitamin E, 1.32 mg vitamin K, 11.6 µg vitamin B₁₂, 14.9 mg niacin, 8.27 mg pantothenic acid, and 2.48 mg riboflavin per kilogram of the complete diet

⁵HiPhos 2700 (DSM Nutritional Products, Inc., Parsippany, NJ), Providing 406.31 Phytase units (FTU)/kg and an estimated release of 0.10% available P.

Table 3.2 Growth and carcass characteristics of barrows fed a conventional swine finishing diet containing 0 mg/kg ractopamine-HCl (**CON**) or a diet formulated to support a finishing barrow fed ractopamine-HCl¹ (**RAC+**) for 35 d

Item	Treatment		SEM	<i>P</i> -value
	CON	RAC+		
Finishing performance				
Initial BW, kg	92.44	91.84	2.18	0.80
Final BW, kg	139.36	141.43	5.80	0.50
ADG, kg	1.18	1.26	0.04	0.15
ADFI, kg	3.82	3.47	0.14	0.03
G:F	0.315	0.365	0.016	< 0.01
Carcass characteristics				
HCW, kg	108.56	107.45	6.22	0.72
Dressing, %	77.69	75.90	1.64	0.33
First rib s.c. fat, cm	3.95	3.71	0.27	0.24
Tenth rib s.c. fat, cm	2.50	2.26	0.18	0.11
Last rib s.c. fat, cm	2.59	2.66	0.17	0.68
Last lumbar s.c. fat, cm	2.07	2.18	0.10	0.45
Loin eye area, cm ²	60.18	62.68	1.26	0.27
Color ²	2.60	2.30	0.15	0.14
Marbling ³	2.00	1.47	0.13	< 0.01

¹10 mg/kg of ractopamine-HCl (Paylean; Elanco Animal Health, Greenfield, IN) fed during the final 35 d of feeding.

²NPPC (1999) Pork Color Standards, 1 = lightest, 6 = darkest.

³NPPC (1999) Pork Marbling Standards, 1 = no marbling, 10 = high marbling.

Table 3.3 Performance test and blood parameters of barrows fed a conventional swine finishing diet containing 0 mg/kg ractopamine-HCl (**CON**) or a diet formulated to support a finishing barrow fed ractopamine-HCl¹ (**RAC+**) for 32 d

Item	Treatment ¹			<i>P</i> -value		
	CON	RAC+	SEM	Treatment	Time	Treatment × Time
Performance test ²						
Average speed, m/s	0.90	0.88	0.42	0.82	--	--
Time to exhaustion, s	395.57	282.40	32.87	<0.01	--	--
Distance to exhaustion, m	563.76	372.53	66.01	<0.01	--	--
Blood parameter, mmol/L						
Plasma glucose			0.23	0.09	<0.01	0.16
Before	4.43	3.33				
After	4.46	3.88				
Plasma lactate			1.13	0.39	<0.01	0.84
Before	3.58	4.38				
After	8.99	10.05				
Plasma glutamate			0.01	0.63	<0.01	0.86
Before	0.09	0.08				
After	0.13	0.13				
Plasma glutamine			0.16	0.63	<0.01	0.16
Before	0.42	0.42				
After	0.38	0.37				

¹10 mg/kg of ractopamine-HCl (Paylean; Elanco Animal Health, Greenfield, IN) fed during the final 35 d of feeding.

²Barrows were removed from pens and individually walked around a 30 m track at 0.79 m/s until subjective exhaustion occurred. Exhaustion was determined as the barrow stopping forward movement (not due to distraction or fear), which resulted in human application of pressure to the rump to resume movement, five times. If a barrow refused to continue after 20 s of encouragement, exhaustion was also designated.

Table 3.4 Muscle fiber characteristics of four muscles in barrows fed a conventional swine finishing diet containing 0 mg/kg ractopamine-HCl (**CON**) or a diet formulated to support a finishing barrow fed ractopamine-HCl¹ (**RAC+**) for 35 d

Item	Muscle ²								SEM	P-value		
	DT		TLH		TFL		ST			TRT ³	Muscle	TRT × Muscle
	CON	RAC+	CON	RAC+	CON	RAC+	CON	RAC+				
Fiber type, %												
Type I	20.41	16.94	15.19	15.57	11.55	6.82	6.70	6.42	1.76	0.07	<0.01	0.30
Type IIA	27.37	27.70	25.60	28.05	28.56	22.08	21.56	23.29	2.46	0.77	0.07	0.10
Type IIX	21.79	20.97	18.29	19.04	17.06	19.16	18.35	14.71	5.26	0.77	0.10	0.50
Type IIB	30.86	34.48	41.02	37.43	42.92	50.03	53.48	55.67	3.53	0.13	<0.01	0.12
Cross-sectional area, μm ²												
Type I	2,771	3,601	3,118	3,445	2,525	2,683	3,876	4,426	508	0.07	<0.01	0.55
Type IIA	3,287	4,240	3,659	4,285	3,474	3,545	4,334	5,085	380	0.03	<0.01	0.36
Type IIX	4,261	5,093	4,977	5,410	5,024	4,891	5,955	6,624	591	0.17	<0.01	0.50
Type IIB	4,747	5,480	5,225	5,672	5,161	5,265	4,722	5,538	535	0.11	0.67	0.63
SDH intensity ⁴ , AU												
Type I	45.50	40.07	42.07	36.56	38.26	39.27	54.83	50.49	9.52	0.21	<0.01	0.64
Type IIA	45.62	45.40	46.97	43.91	42.68	44.71	56.79	58.87	10.16	0.93	<0.01	0.78
Type IIX	66.90	60.90	64.45	57.95	61.64	61.46	73.39	73.00	15.85	0.22	<0.01	0.65
Type IIB	78.17	75.30	82.35	70.58	75.61	77.80	82.26	81.14	20.75	0.27	0.35	0.21
Capillary density												
Ratio ⁵	1.44	1.88	1.50	2.04	1.16	1.60	1.50	2.21	0.29	0.03	0.17	0.92

¹10 mg/kg of ractopamine-HCl (Paylean; Elanco Animal Health, Greenfield, IN) fed during the final 35 d of feeding.

²DT = *Deltoideus*, TLH = *Triceps brachii lateral head*, TFL = *Tensor fasciae latae*, and ST = *Semitendinosus*.

³TRT = treatment.

⁴Succinate dehydrogenase; 0 = most intense staining, 250 = least intense staining; AU = arbitrary units.

⁵Capillary ratio was determined by dividing the total number of capillaries by the total number of muscle fibers.

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Appendix A - Representative photographs depicting surface electromyography (EMG) sensors used during a performance test

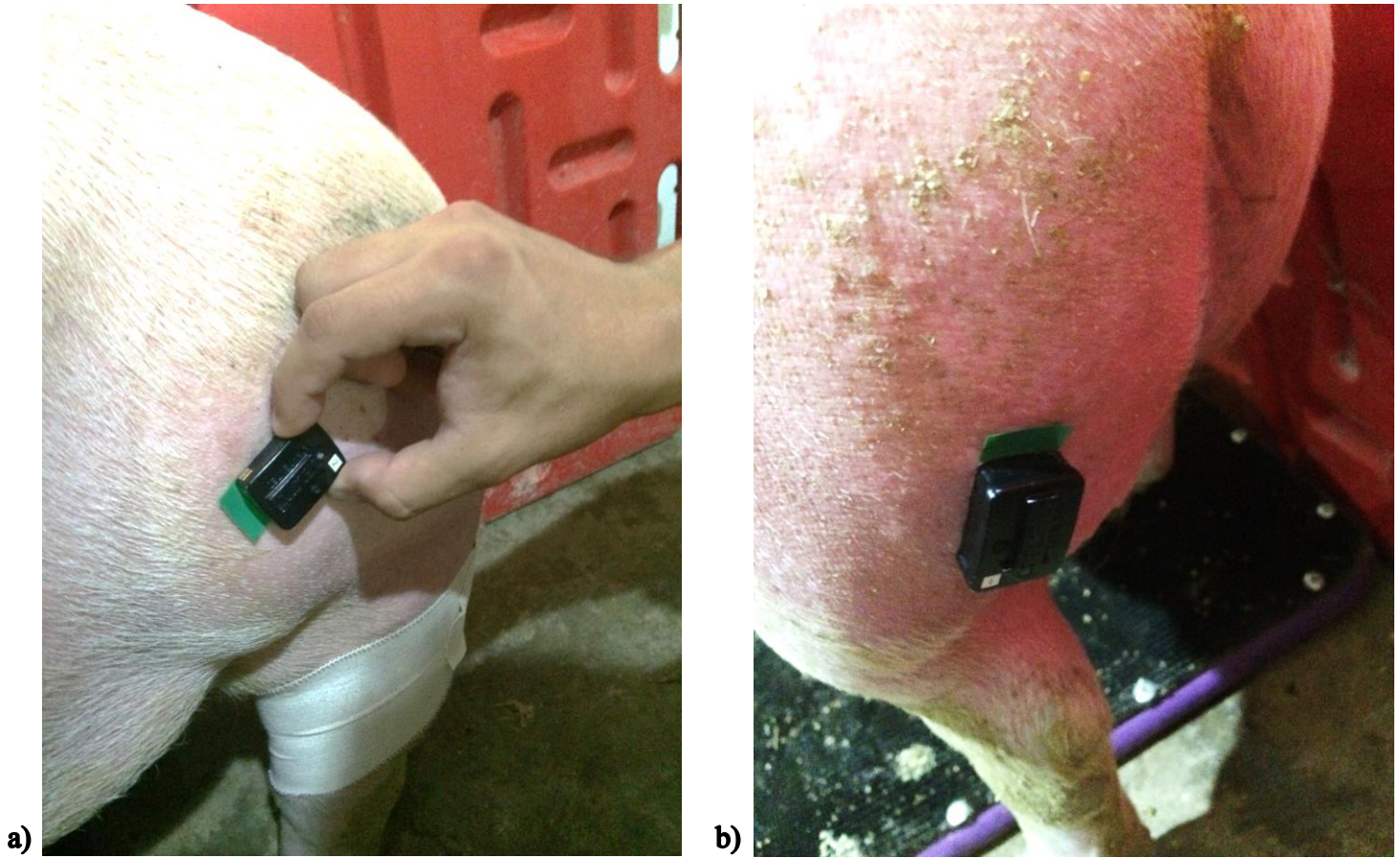


Figure A.1 Representative photographs depicting surface electromyography (EMG) sensors on the a) *Tensor fascia latae* (TFL) and b) *Semitendinosus* (ST) during a performance test to determine exhaustion in barrows fed either a conventional swine finishing diet containing 0 mg/kg ractopamine-HCl (CON, Paylean; Elanco Animal Health, Greenfield, IN) or a diet formulated to support a finishing barrow fed ractopamine-HCl (RAC) for 32 d. Using hair clippers (305 Speed Feed Trim Kit; Laube Co., Inc., Oxnard, CA), the hair covering muscles on the left side of the barrow was trimmed. The shaved areas were cleaned with 70% ethanol, air dried, and wireless EMG (Tringo EMG, Delsys, Natick, MA) electrodes were affixed to each muscle so that the 4 contact bars arranged in a 2×2 orientation were placed perpendicular to the orientation of the muscle fibers.

**Appendix B - Representative photograph depicting
electromyography (EMG) sensor placement during a performance
test**

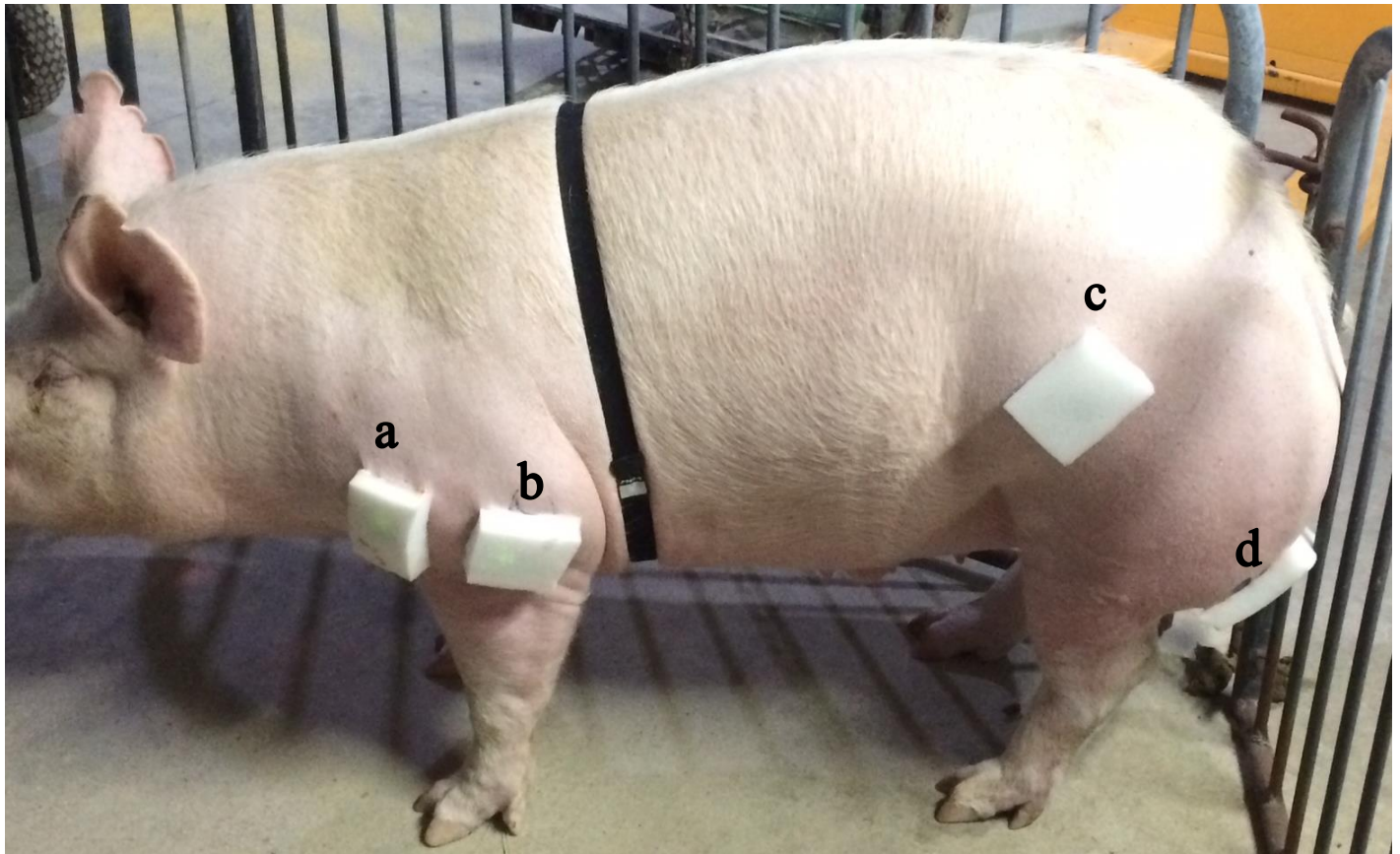


Figure B.1 Representative photograph depicting surface electromyography (EMG) sensor placement on 4 main locomotor muscles including the a) *Deltodieus* (DT), b) *Triceps brachii lateral head* (TLH), c) *Tensor fascia latae* (TFL), and d) *Semitendinosus* (ST) during a performance test to determine exhaustion in barrows fed either a conventional swine finishing diet containing 0 mg/kg ractopamine-HCl (CON, Paylean; Elanco Animal Health, Geenfield, IN) or a diet formulated to support a finishing barrow fed ractopamine-HCl (RAC) for 32 d. Using hair clippers (305 Speed Feed Trim Kit; Laube Co., Inc., Oxnard, CA), the hair covering muscles on the left side of the barrow was trimmed. The shaved areas were cleaned with 70% ethanol, air dried, and wireless EMG (Tringo EMG, Delsys, Natick, MA) electrodes were affixed to each muscle so that the 4 contact bars arranged in a 2 × 2 orientation were placed perpendicular to the orientation of the muscle fibers.

Appendix C - Representative photograph depicting the circular track used during a performance test



Figure C.1 Representative photograph depicting the 30 m track that barrows were individually briskly walked around during a performance test until exhaustion occurred in barrows fed either a conventional swine finishing diet containing 0 mg/kg ractopamine-HCl (**CON**, Paylean; Elanco Animal Health, Greenfield, IN) or a diet formulated to support a finishing barrow fed ractopamine-HCl (**RAC**) for 32 d. Exhaustion was determined as the barrow stopping forward movement (not due to distraction or fear), which resulted in human application of pressure to the rump to resume movement, five times. If a barrow refused to continue after 20 s of encouragement, exhaustion was also designated.

Appendix D - Immunofluorescence staining protocol: BF-F3, SC-71, BA-D5, and dystrophin on porcine muscle cryosections

Blocking Solution

5% Horse serum (**HS**)/0.2% Triton-X-100 in PBS (pH of 7.4)

*Make all primary and secondary dilutions with blocking solution

Primary Antibodies

1) Dystrophin (Prod # PA137587, Thermo Fisher)

a) Pierce Anti-dystrophin rabbit polyclonal

b) Dilution of 1:500

2) BF-F3 (mouse IgM, DHSB BF-35)

a) Myosin heavy chain type IIB

b) Dilution of 1:10

3) SC-71 (mouse IgG1, DHSB SC-71)

a) Myosin heavy chain type IIA

b) Dilution of 1:10

4) BA-D5 (mouse IgG2b DHSB BA-D5)

a) Myosin heavy chain type I

b) Dilution of 1:10

Secondary Antibodies

1) Alexa-Fluor 488 goat anti-mouse IgM (Cat # A-21121, Life Technologies)

a) Dilution of 1:1,000

2) Alexa-Fluor 594 goat anti-mouse IgG1 (Cat # A-21125, Life Technologies)

a) Dilution of 1:1,000

- 3) Alexa-Fluor 633 goat anti-mouse IgG2b (Cat # A-21146, Life Technologies)
 - a) Dilution of 1:1,000
- 4) Alexa-Fluor 488 goat anti-rabbit IgG H&L (Cat # A-11008, Life Technologies)
 - a) Dilution of 1:1,000
- 5) Alexa-Fluor 594 goat anti-rabbit IgG H&L (Cat # A-11012, Life Technologies)
 - a) Dilution of 1:1,000
- 6) Hoescht dye (Cat # 33342, Life Technologies)
 - a) Dilution of 1:1,000

Staining Procedure

- 1) Use a pap pen to make a hydrophobic ring around the edge of each slide while the slide is dry
- 2) To block non-specific antigen binding, incubate cryosections with blocking solution (100 μ l per section) at room temperature in a humidified box for 30 min
 - a) Use a tip box that has the top wrapped in foil and a wet paper towel in the bottom to provide a humid environment
- 3) Remove blocking solution from each slide using a pipette tip in the corner of each slide
- 4) Add primary antibody solution (100 μ l per section) and incubate at room temperature in a humidified box for 1 h
 - a) Primary antibodies can be combined into a single solution
- 5) Rinse with PBS 3 \times for 5 min each
- 6) Add secondary antibodies and Hoescht dye solution (100 μ l per section) and incubate at room temperature in a humidified box for 30 min
 - a) Protect slides from light for the remainder of procedure
- 7) Rinse with PBS 3 \times for 5 min each

8) Coverslip with 9:1 glycerol/PBS solution and fingernail polish the edges of the coverslip to seal

a) Once dry, slides can be stored at room temperature for 7-14 d if protected from light

Appendix E - Succinate dehydrogenase (SDH) staining protocol on porcine muscle cryosections

Solutions

- 1) Phosphate buffer stock solution (100 mL) keep for up to 1 month at room temperature
 - a) Make each separately and then mix together:
 - i) 0.195g Potassium phosphate monohydrate (KH_2PO_4) in 12 mL MQ water
 - ii) 10.99 g of Disodium hydrogen phosphate (Na_2HPO_4) in 88 mL MQ water
- 2) Sodium succinate stock solution (100 mL) keep for up to 1 month at room temperature
 - a) 2.7014 g of Sodium succinate dibasic hexahydrate in 100 mL of MQ water
- 3) Nitroblue tetrazoliumand (20 mL) make fresh each time
 - a) .02 g Nitroblue tetrazoliumand (NBT) in 20 mL of MQ water

Staining Procedure

- 1) Make incubation solution
 - a) Make 20 mL NBT
 - i) Mix 20 mL NBT, 10 mL phosphate buffer stock solution, and 10 mL sodium succinate stock solution
- 2) Place incubation solution in staining jar and pre-warm in 37°C water bath for 30 min
- 3) Let slides warm to room temp for 5 min
- 4) Place slides in incubation solution and incubate in 37°C water bath for 1-2 h
- 5) Wash in MQ water 3× for 1 min each
- 6) Wipe slide dry with kimwipe (do not touch sections)
- 7) Add 1 drop of 9:1 glycerol: 1× PBS to each section

- 9) Coverslip with 9:1 glycerol/PBS solution and fingernail polish the edges of the coverslip to seal

Appendix F - Representative photomicrographs of succinate dehydrogenase staining pattern of porcine *Triceps brachii lateral head* (TLH) muscle

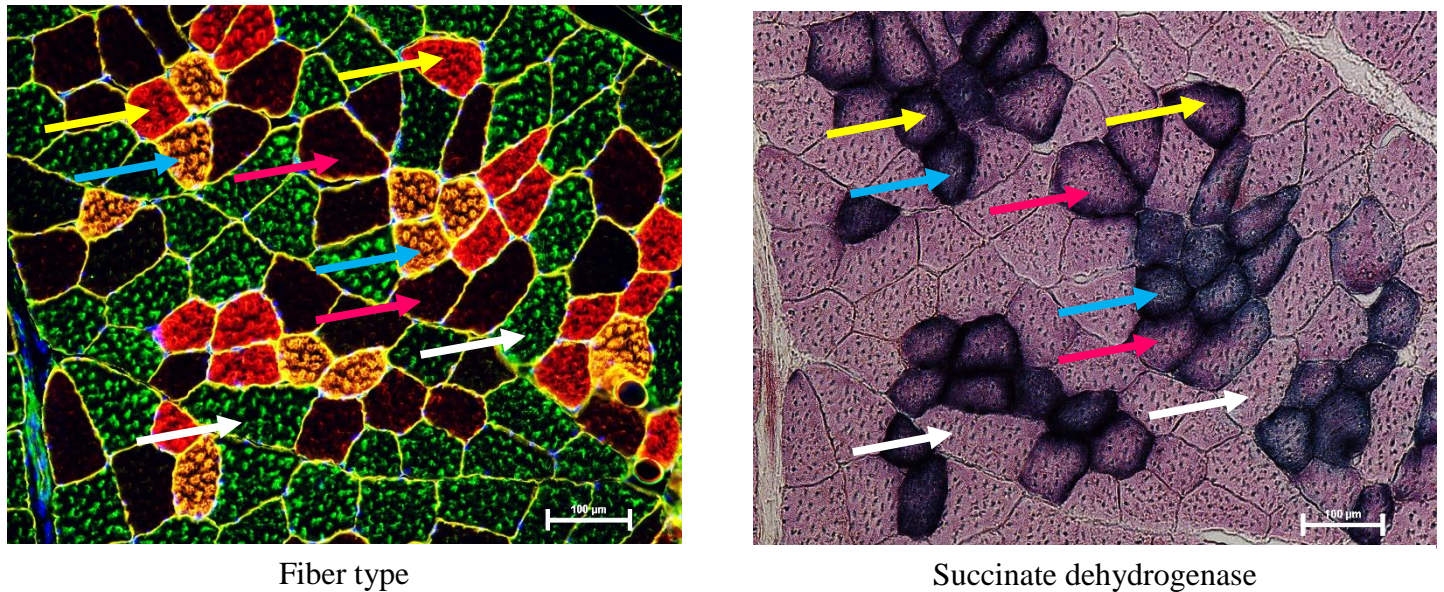


Figure F.1 Representative photomicrographs of succinate dehydrogenase fiber type staining pattern of porcine *Triceps lateral head muscle* (TLH). A serial section was immunohistologically stained for fiber type identification. Fibers that stained positive for the BA-D5 antibody were categorized as type I fibers (Blue arrows), fibers that stained positive for the SC-71 antibody only were categorized as type IIA fibers (Yellow arrows), fibers that stained positive for the BF-F3 antibody only were categorized as type IIB fibers (White arrows), and fibers that stained positive for both SC-71 and BF-F3 were categorized as type IIX fibers (Pink arrows; Paulk et al., 2014). Scale bars = 100 µm. Intensity scale: 0 = most intense staining, 250 = least intense staining.

Appendix G - Immunofluorescence staining protocol: PECAM-1 and dystrophin on porcine muscle cryosections

Blocking Solution

5% Horse serum/0.2% Triton-X-100 in PBS (pH of 7.4)

*Make all primary and secondary dilutions with blocking solution

Primary Antibodies

- 1) Dystrophin (Prod # PA137587, Thermo Fisher)
 - a. Pierce Anti-dystrophin rabbit polyclonal
 - b. Dilution of 1:500
- 2) PECAM (mouse IgG2a, Prod # 59937, Santa Cruz Biotechnology)
 - a. Anti-PECAM-1 Mouse monoclonal
 - b. Dilution of 1:50

Secondary Antibodies

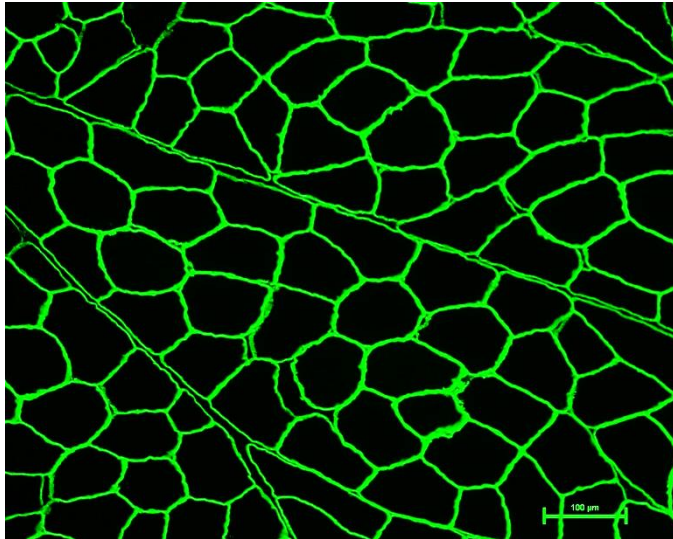
- 1) Alexa-Fluor 594 goat anti-mouse IgG H&L (Cat # A-21125, Life Technologies)
 - a. Dilution of 1:1,000
- 2) Alexa-Fluor 488 goat anti-rabbit IgG H&L (Cat # A-11008, Life Technologies)
 - a. Dilution of 1:1,000
- 3) Hoescht dye (Cat # 33342, Life Technologies)
 - a. Dilution of 1:1,000

Staining Procedure

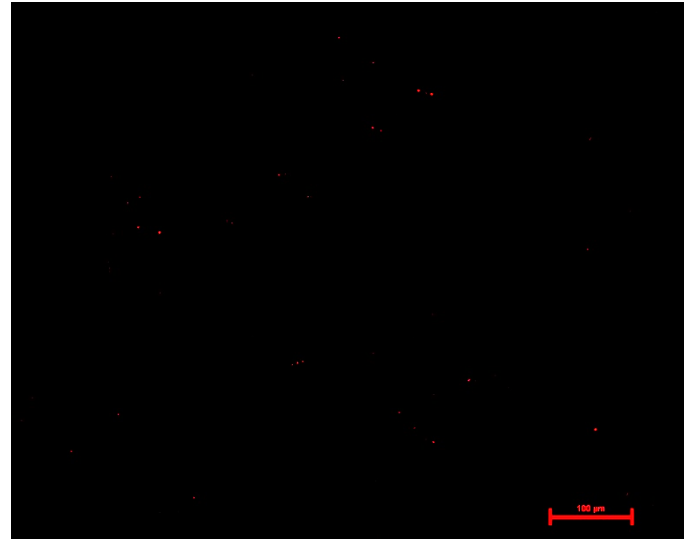
- 1) Use a pap pen to make a hydrophobic ring around the edge of each slide while to slide is dry

- 2) To block non-specific antigen binding, incubate cryosections with blocking solution (100 μ l per section) at room temperature in a humidified box for 30 min
 - a. Use a tip box that has the top wrapped in foil and a wet paper towel in the bottom to provide a humid environment
- 3) Remove blocking solution from each slide using a pipette tip in the corner of each slide
- 4) Add primary antibody solution (100 μ l per section) and incubate at room temperature in a humidified box for 1 h
 - a. Primary antibodies can be combined into a single solution
- 5) Rinse with PBS 3 \times for 5 min each
- 6) Add secondary antibodies and Hoescht dye solution (100 μ l per section) and incubate at room temperature in a humidified box for 30 min
 - a. Protect slides from light for the remainder of procedure
- 7) Rinse with PBS 3 \times for 5 min each
- 8) Coverslip with 9:1 glycerol/PBS solution and fingernail polish the edges of the coverslip to seal
 - a. Once dry, slides can be stored at room temperature for 7-14 d if protected from light

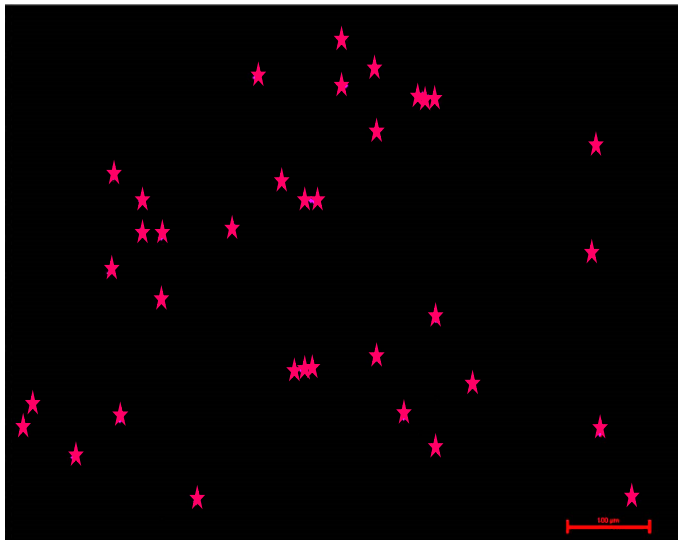
**Appendix H - Representative photomicrographs of
immunohistological capillary staining pattern of porcine *Deltoideus*
(DT) muscle**



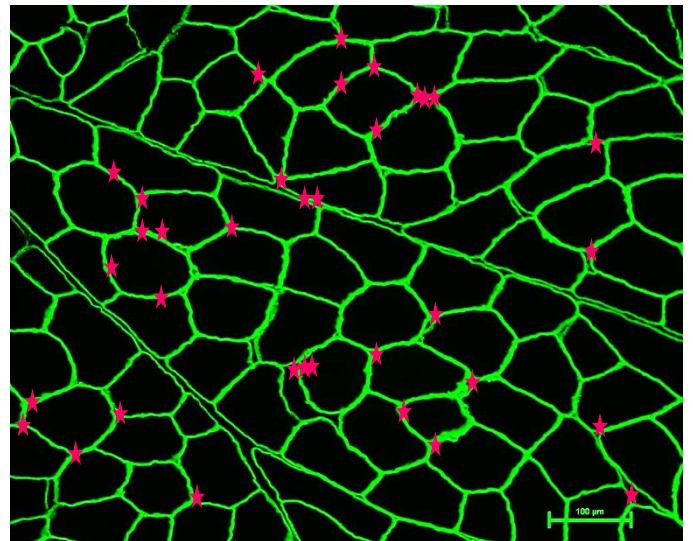
Dystrophin (Cell outlines)



PECAM (Capillaries)



Marked capillaries



Dystrophin and marked capillaries

Figure H.1 Representative photomicrographs of immunohistological capillary staining pattern of porcine *Deltoideus* (DT) muscle. Dystrophin antibody was used to stain the outline of muscle fibers (green outline) and PECAM was used to stain for capillaries (marked by pink stars). Capillary density was determined by dividing the total number of capillaries by the total number of muscle fibers within a pictograph.